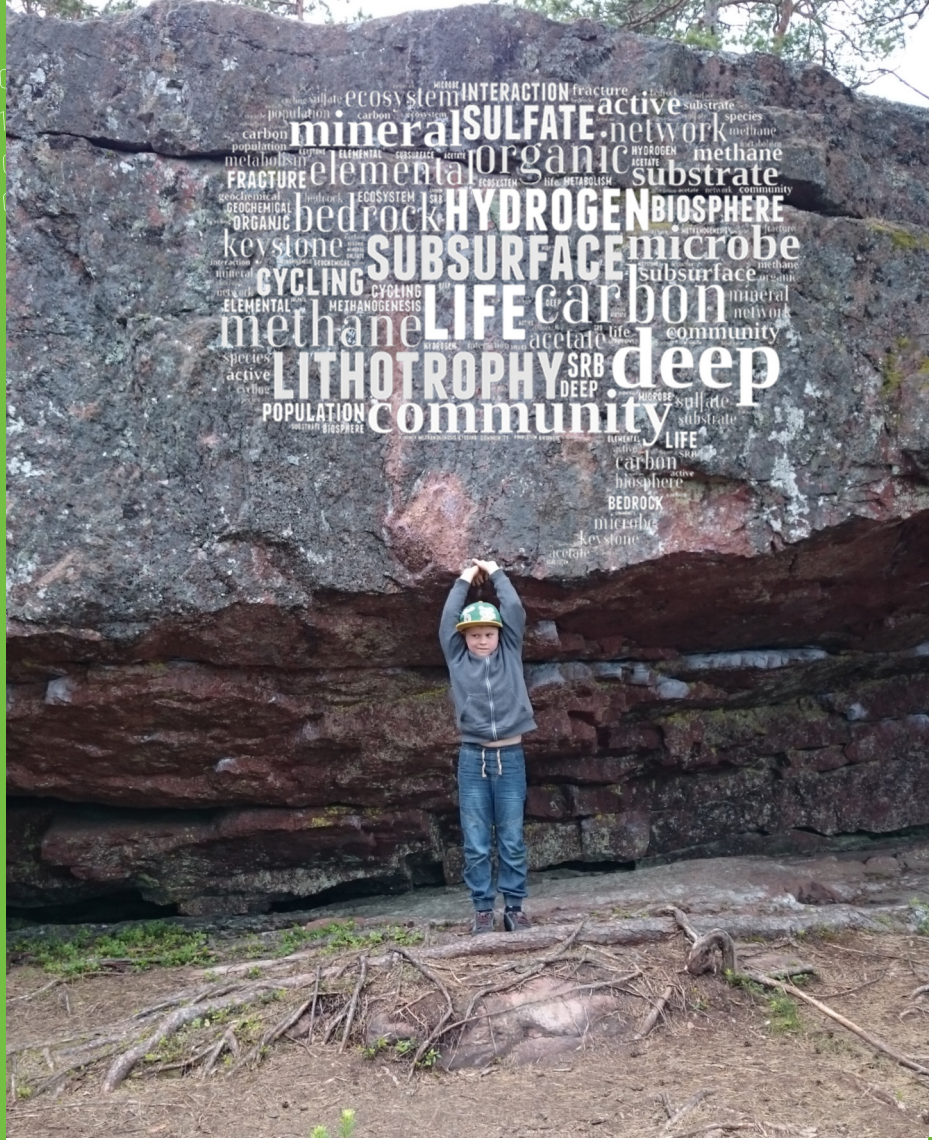

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Dissertation
116

Microbial ecology and functionality in deep Fennoscandian crystalline bedrock biosphere

Lotta Purkamo

VTT SCIENCE 116

Microbial ecology and functionality in deep Fennoscandian crystalline bedrock biosphere

Lotta Purkamo

VTT Technical Research Centre of Finland Ltd

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Preface

This thesis consists of studies conducted at the VTT Technical Research Centre of Finland Ltd during 2009–2015. The research projects were funded by the Academy of Finland, Finnish Research Programme for Nuclear Waste Management (KYT), Foundation for Research of Natural Resources in Finland and Kone Foundation. University of Helsinki's Dissertation completion grant enabled the final effort in composing the thesis.

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As the note from fictional icelandic alchemist Arne Saknussem said in Jules Verne's book *A journey to the Centre of the Earth*: "Descend, bold traveller, to the centre of the earth. I did it."

Kirkkonummi, November 2015
Lotta Purkamo

Academic dissertation

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List of publications

This thesis is based on the following publications, which are referred in the text as Article I, Article II and Article III.

I Purkamo L, Bomberg M, Nyyssönen M, Kukkonen I, Ahonen L, Kietäväinen R, Itävaara M. Dissecting the deep biosphere: retrieving authentic microbial communities from packer-isolated deep crystalline bedrock fracture zones. *FEMS Microbiol Ecol.* 2013, 85(2):324–37. doi: 10.1111/1574-6941.12126.

II Purkamo L, Bomberg M, Nyyssönen M, Kukkonen I, Ahonen L, Itävaara M. Heterotrophic communities supplied by ancient organic carbon predominate in deep Fennoscandian bedrock fluids. *Microb Ecol*. 2015, 69(2):319–32. doi:10.1007/s00248-014-0490-6.

III Purkamo L, Bomberg M, Kietäväinen R, Salavirta H, Nyyssönen M, Nuppunen-Puputti M, Ahonen L, Kukkonen I, Itävaara M. The keystone species of Precambrian deep bedrock biosphere belong to *Burkholderiales* and *Clostridiales.* Accepted to *Biogeosciences Discussions*.

Author's contributions

I Lotta Purkamo took part in planning of the experiments and collecting the samples. She carried out molecular biological work and took part in microscopy analyses. She did the cluster analysis of DGGE and sequence analyses, interpreted the results, wrote the paper and is the corresponding author.

II Lotta Purkamo took part in planning the experiments and participated in the molecular biological analyses. She carried out sequence and statistical analyses, interpreted the results and wrote the paper. She is the corresponding author.

III Lotta Purkamo planned the experiment, took part in sampling and laboratory work, carried out the community analyses, bioinformatics and network analysis. She interpreted the results, wrote the paper and is the corresponding author.

Supporting publications

Kietäväinen, R and Purkamo L. The origin, source, and cycling of methane in deep crystalline rock biosphere. *Front. Microbiol.* 2015. (6) 725. doi.org/10.3389/fmicb. 2015.00725

Contents

List of abbreviations

ANME	anaerobic methane oxidizing archaea
ATP	adenosine triphosphate
CCA	canonical correspondence analysis
CRB	Columbia River Basalts
DAPI	4'-6-diamidino-2-phenylindole
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
FID	flame ionization detector
FTT	Fischer-Tropsch type
Ga	Giga-annuum
HTP	high throughput (sequencing)
ICP-MS	inductively coupled plasma mass spectrometry
ICP-OES	inductively coupled plasma optical emission spectrometry
MPN	most probable number
MQ	Milli–Q (ultrapure water)
NSTI	nearest sequenced taxon index
PCR	polymerase chain reaction
PLFA	phospholipid fatty acid
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RO	reverse osmosis
rRNA	ribosomal ribonucleic acid

SLiME	subsurface lithotrophic microbial ecosystem
SMTZ	sulfate-methane transition zone
SRB	sulfate reducing bacteria
TCD	thermal conductivity detector
TDS	total dissolved solids
TEA	terminal electron acceptor
WGA	whole genome amplification

1. Introduction

1.1 The deep biosphere

The deep biosphere can be defined as **an ecosystem of organisms and their living space** extending from several tens of meters to several kilometers in the Earth's crust (Hoehler and Jørgensen 2013). Thomas Gold (1992) suggested that if the estimated pore space of the land areas down to 5 km depth of the Earth's crust would be filled with water and 1% of this volume would be microbial biomass, this biomass would be sufficient to cover Earth's land surface with a 1.5 m thick layer. The anthropogenic use of deep terrestrial subsurface as a source of material, energy, or storage space has triggered the motivation to study the deep biosphere of these environments. These operations include oil and hydrocarbon recovery and storing of the crude or refined materials of these processes, CO_2 sequestration, traditional mining activities as well as modern biohydrometallurgical processes, geothermal heat and energy facilities and nuclear waste repositories (e.g. Pedersen 1996, Stroes-Gascoine and West 1997, Head et al. 2003, Mitchell et al. 2008, Das et al. 2011, Nyyssönen et al. 2012, Gniese et al. 2014, Wouters et al. 2013). Microbiological risks for both the financial profitability of these operations and for the environmental safety where such operations are conducted are present. Petroleum reservoirs are susceptible for microbial biodegradation of crude oil, resulting in alteration of the hydrocarbon composition of the reservoir and excess methane production (Jones et al. 2008). A major risk for many operations is microbially induced corrosion (Javaherdashti 2011, Gniese et al. 2014, Lerm et al. 2013). Sulfate-reducing bacteria are key organisms acting in microbially induced corrosion processes, and thus have been under study also in many deep subsurface studies that are aiming to characterize microbial risks of for example underground storage of gas and nuclear wastes (e.g. Pedersen et al. 2008, Pedersen 2012a,b, Nyyssönen et al. 2012, Gniese et al. 2014, Rajala et al. 2015). Another severe challenge for example in geothermal energy operations is scaling of the equipment surfaces induced by thick corrosion products or insoluble minerals. Usually scaling becomes more severe as temperature decreases in the system (Valdez et al. 2009, Lerm et al. 2013).

However, some technologies may harness the microbial power for economical profit and restoration of contaminated environments. The adverse effect of micro-

bial biodegradation of for example crude oil can be useful for bioremediation purposes (eg. Scow and Hicks 2005, Meckenstock et al. 2015), it may also provide a way of recovering energy from oil fields with environmentally less harmful technologies, in form of methane (Jones et al. 2008). Uranium or heavy metal - contaminated mining sites can be bioremediated by the help of sulfate reducers by precipitation of metal sulfides through sulfate reduction (Lloyd and Lovley 2001). The in-situ mining of low-grade ores exploiting the known microbial processes in controlled conditions can be utilized to extract valuable metals (Das et al. 2011).

Beyond the anthropogenic point of view of economical utilization of the deep subsurface and understanding of the microbial risks, deep biosphere studies will provide a window to the past and even to the other worlds. The deep subsurface may have been the only refuge for life during the early history of Earth when meteoric impacts have sterilized the surface regularly (Cockell et al. 2012). In addition, understanding diversity, richness and functionality of the microbial communities in life-sustaining environments on Earth facilitate our ability to interpreting the possibility of life on other planetary bodies (Amend and Teske 2005).

The first attempt to evaluate the magnitude of the biomass in Earth's deep biosphere was made almost two decades ago by Whitman et al. (1998). They suggested that the deep subsurface would host 3.8×10^{30} microbial cells or 5×10^{17} g of carbon in microbial biomass. The recent reassessment of the amount of biomass in deep continental biosphere supported the early estimates. The biomass of the deep continental biosphere can be up to 19% of the total biomass on Earth, and is equal or even somewhat greater than the biomass of the deep marine subsurface (Hinrichs and Inagaki 2012, Kallmeyer et al. 2012, McMahon and Parnell 2014).

1.2 Environmental conditions affecting the existence of life in deep subsurface

Ultimately four factors must be met for microbial colonization of a certain environment: space, water, suitable temperature and material for energy and cellular building blocks. Habitat volume for organisms in the deep biosphere depends on the geological history of the subsurface. Sedimentary rocks are considered more porous than igneous rocks. However, igneous or metamorphic crystalline rocks can be fractured and shattered, thus rubble and fracture zones provide substantial living space for deep subsurface microbial communities (Stevens 1997).

Water is commonly present in the subsurface in pores, fissures and in larger fracture zones, and is not limiting microbial life. However, the very large solid rock vs. water ratio is limiting the living space of microbes in deep biosphere (Pedersen 2000). In shallower depths, water circulates slowly and mixing with the meteoric water is likely to happen, but with decreasing hydraulic conductivity deeper in a rock formation, water becomes stagnant resulting in very long residence times (Holland et al. 2013, Kietäväinen et al. 2014).

While water availability is not the limiting factor for life in the deep subsurface, the temperature of the environment may be. The current estimate for temperature limit of life is 122 °C and the record holder is a hyperthermophilic archaeon *Methanopyrus kandleri* (Takai et al. 2008). The depth range, where temperature exceeds this limit, is however very broad. It can vary from the surface of the sea floor at the tectonic plate boundaries or hydrothermal fields to depth of 10 km and even deeper in stable sedimentary rock formations (Pedersen 2000). To date, the deepest continental site where bacteria have been detected is in Gravberg, Sweden, where glucose-fermenting thermophilic bacteria originating from a depth of over 5.2 km were isolated (Szewzyk et al. 1994).

CHNOPS – Carbon, hydrogen, nitrogen, oxygen, phosphorus and sulfur are the elements of life, the Lego blocks that all living organisms are made of. Carbon forms the chemical backbone of all organic compounds and the versatility of forming single, double or triple bonds, long chains, flexible ring structures and stable complex molecules makes carbon so special. Three vital components of a cell are based on carbon chains: DNA, membranes and proteins (Cockell and Nixon, 2013). Deep bedrock environments are commonly oligotrophic due to their spatial isolation and the usually nonexistent connection to meteoric water cycle (Lovley and Chapelle 1995). If deep subsurface fractures are connected with water cycle on the surface and shallow groundwater systems, the dissolved organic carbon is typically consumed already in the shallow depths leaving only the most recalcitrant material left for the deep ecosystems (Kotelnikova 2002). Therefore, life in the deep subsurface is defined by the scarcity of photosynthetically produced energy and carbon and other environmental factors, such as low concentration or absence of oxygen, pH extremes, high salinity and pressure.

In addition to above mentioned, life in the deep subsurface has to be adapted to other extreme conditions. Extremophiles, i.e. microbes living in extreme environments, have developed numerous mechanisms coping with nutrient deprivation and limited energy availability in addition to high pressure, temperature, extreme alkaline or acidic conditions and even metal toxicity and radioactivity (Pikuta et al. 2007).

1.3 Energetics in the deep biosphere

The primary energy source in the deep biosphere is proposed to be geochemical. This is obvious, because several deep subsurface systems appear to be completely detached from the ultimate source of energy on the surface, namely sunlight (Gold 1992, Amend and Teske 2005, Colwell and D'Hondt 2013). The concept of the thermodynamic tower or ladder, where electron accepting-processes are arranged hierarchically from most energy yielding towards less energetic processes has been used in many environmental studies to describe the microbial zonation of for example pristine and contaminated aquifers (Christensen et al. 1994, Lovley and Chapelle 1995, Meckenstock et al. 2015). However, ecological and physiological factors control the distribution of microbial life in the deep sub-

surface in addition the thermodynamic ladder (Bethke et al. 2011). Aerobic respiration, in which photosynthetically produced oxygen is the terminal electron acceptor (TEA), is the most energy-yielding process. Although oxygen can diffuse to some extent to the subsurface, it is most likely consumed quickly as it enters the subsurface (Lovley and Chapelle 1995). Yet, oxygen may be produced in deep crustal surroundings during radiolysis of H_2O in addition to H_2 and thus may facilitate aerobic growth of microbes in deep systems (Pedersen 2000, Lin et al. 2005a,b). Nevertheless, the deep biosphere is mostly dependent on other terminal electron acceptors in lower rungs of the thermodynamic ladder, including nitrate, iron, manganese, sulfate and carbon compounds.

Energy acquisition in deep, anoxygenic subsurface can be based on anaerobic respiration and fermentation. In anaerobic respiration, an organism uses inorganic or organic compounds other than oxygen as electron acceptors and ATP is produced via proton motive force in a process called oxidative phosphorylation. This is called chemotrophy as the electron donors and acceptors are chemical, in contrast to phototrophy, where light is the source of energy. Chemotrophs are divided further into two groups. Chemoorganotrophs use organic compounds as electron donors and can utilize the same material for biosynthesis, while chemolithotrophs can only use inorganic compounds as their electron donors and thus, have to gain their carbon for biosynthesis elsewhere. Autotrophic carbon fixation is characteristic to chemolithotrophs, meaning that carbon is assimilated in the form of CO_2 and thus these organisms are sometimes referred as chemolithoautotrophs (Kim and Gadd 2008).

Fermentation is another anaerobic process, where cells use organic material for production of energy and biosynthesis. Substrate variety for fermentation is wide, ranging from carbohydrates to organic acids, amino acids and nucleic acid bases. ATP is produced via substrate-level phosphorylation in contrast to anaerobic respiration. Fermentation is energetically in the lower rungs of the thermodynamic ladder as it occurs at very low redox potentials (e.g. Amend and Teske 2005). Organic matter fermenters enable the growth of other microbial groups by providing acetate or CO_2 to chemoorganotrophs and chemolithotrophs, respectively. H_2 is produced in many fermentation processes and is a prominent electron donor for chemolithotrophs in the deep subsurface. However, if the partial pressure of H_2 exceeds a certain level, fermentation will be inhibited. Thus, in subsurface, radiolytically formed hydrogen (see next paragraph) can inhibit fermentative growth (Lin et al. 2005b).

Because organic compounds for fermentation and anaerobic respiration are scarcely available in deep crystalline bedrock environments, chemolithotrophy is traditionally considered to be the prevailing metabolism and CO_2 the predominant source of carbon in deep bedrock (Fredrickson et al. 1997, Fredrickson and Balkwill 2006, McCollom and Amend 2005). In chemolithoautotrophy, both the energy source and the electron sink i.e. the TEA is inorganic and biomass is produced from inorganic carbon (e.g. Stevens 1997). As mentioned, a useful source of reducing power for chemotrophic microbes is H_2, which is abundantly available in many crystalline bedrock systems (e.g. Pedersen 1997, 2000, Nealson et al. 2005,

Sherwood Lollar et al. 2014, Kietäväinen et al. 2014). Thus, H_2 is considered the sole energy source for photosynthesis-independent hydrogen-driven lithotrophic microbial ecosystems (SLiMEs) in deep subsurface (Amend and Teske 2005, McCollom and Amend 2005, Nealson et al. 2005). Geochemical hydrogen can be produced in deep terrestrial bedrock via abiotic water-mineral interactions, such as serpentinization occurring in ultramafic rock systems or by dissociation of water molecules with the energy released in radioactive decay, or can be derived from deeper, hotter parts of the crust via gas flux (e.g. Pedersen 2000, Nealson et al. 2005, Lin et al. 2005a,b, McCollom and Bach 2009). In addition, some microbes are able to mediate H_2 production from minerals, thus securing a continuous supply of energy for themselves (Parkes et al. 2011).

However, it should be noted that the theoretic energetic potential is not usually applicable as in natural systems. Therefore it would be useful to weigh the energetics in reference to the availability of reactants in order to provide a more realistic view of the potential energy metabolisms in the deep biosphere (Osburn et al. 2014).

Finally, the growth rates in deep subsurface are extremely low. Due to the extremely low flux of energy and nutrients, estimates on generation time reach 1000 years (Jørgensen and D'Hondt 2006). Thus, microbial communities in deep subsurface are likely adapted to the low energy flux and prefer to use such mechanisms in their cell metabolism that save energy (Hoehler and Jørgensen, 2013). Recently, a minimum flux of energy, i.e. power requirement for a cell to remain viable was estimated to be 1×10^{-15} μW $cell^{-1}$ (LaRowe and Amend 2015).

1.4 Geochemical sources of carbon

On the surface of the Earth, most of the primary production relies on photosynthesis, in which carbon dioxide from the atmosphere is converted into sugars by photoautotrophs, such as green plants. After the postulation of the so-called hydrogen-driven deep biosphere (Gold 1992), several studies have suggested that autotrophic H_2-utilizing microbes are responsible for primary production in the deep subsurface (Stevens and McKinley 1995, Pedersen 1997, 2000). These autotrophic chemolithotrophs are using carbon dioxide found usually in dissolved form as HCO_3^- in deep groundwater (Frape et al. 2013) (**Table 1**). Carbon dioxide is formed in the mantle or in lower crust from where it is outgassed to shallower depths of the crust during various geological processes, such as metamorphism, mountain building and volcanic activities (Kerrick 2001) (**Figure 1**).

However, primary production in deep biosphere has also been proposed to rely on organic molecules synthetized abiotically in geochemical processes from CO_2 and H_2 (Amend and Teske 2005, Schrenk et al. 2013). During serpentinization of ophiolitic rocks, water interacts with ferrous iron-rich minerals of ultramafic rocks and H_2 is produced (McCollom and Bach 2009). Serpentinization provides a continuous flux of H_2 resulting in a highly reducing environment where methane, C_2+ hydrocarbons and minor amounts of organic acids are formed from CO_2

(McCollom and Seewald 2001, Lang et al. 2010, Russell et al. 2010, Szponar et al. 2013) (**Figure 1**). While many chemolithotrophs can use H_2 as an energy source, H_2 produced during serpentinization enhances the reduction of CO_2 to methane and other organic molecules. Therefore, serpentinization may enable also chemoorganotrophic microbial life (Russell et al. 2010, Schrenk et al. 2013).

In addition, small alkanes and alkenes are typically formed in laboratory experiments of Fischer-Tropsch type (FTT) synthesis (Taran et al. 2007, McCollom et al. 2010, Zhang et al. 2013). Proskurowski et al. (2008) demonstrated that synthesis of small hydrocarbons is possible in FTT reactions *in situ* in natural hydrothermal environment supplied by ultramafic crust. Moreover, methane and other light hydrocarbons can be produced abiotically in either high temperature (> 500 °C) reactions in the mantle as well as in gas-water-rock reactions in the crust, at lower temperatures (25–500 °C) (Etiope and Sherwood Lollar 2013, McCollom 2013). These organic carbon molecules are prominent carbon and energy source for heterotrophs, which have been discovered in many deep crystalline rock sites (see Section 1.7).

Microbial communities in rock formations can also be fuelled by ancient photosynthetically produced organic carbon preserved into minerals such as black shale (Krumholz et al. 1997, Petsch et al. 2001). Interlayers of metamorphosed black schist can contain refractory organic material also in Outokumpu (Taran et al. 2011), and thus provide one plausible carbon source for the deep bedrock biosphere.

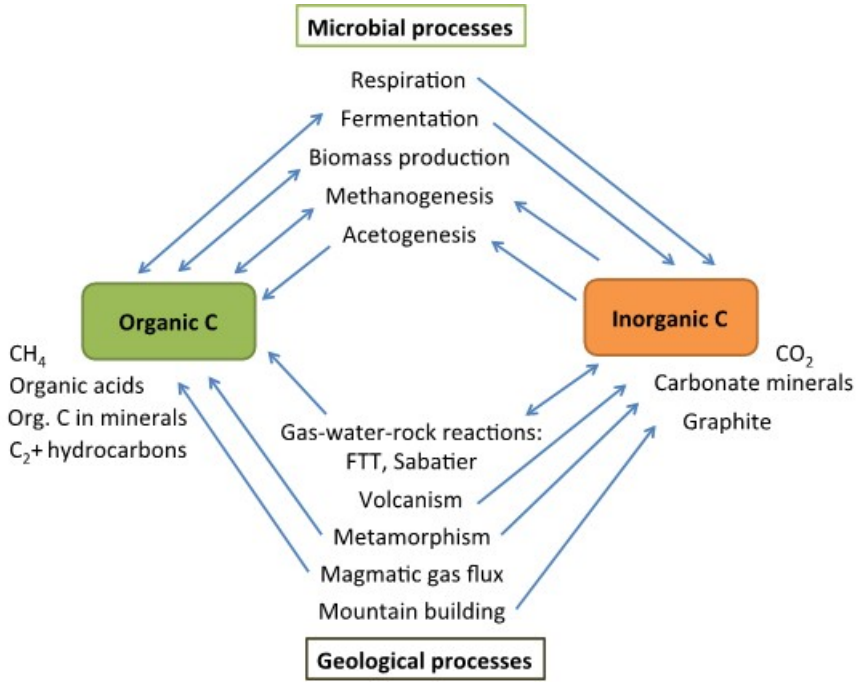

Figure 1. The geological and biological sources and cycling of carbon in deep crystalline bedrock.

1.5 Microbial ecology in the deep subsurface

As the amount of microbial biomass in the subsurface is significant, these microbes likely have functional potential beyond our current knowledge. Therefore, microbial ecology studies are needed for understanding and possible subsequent harnessing of this functionality to our benefit. Additionally, these studies enable the microbial risk assessment of many technological applications using the deep bedrock. Moreover, the microbial biomass in the deep subsurface presumably has a significant role in global elemental cycling.

Microbial ecology research focuses on diversity, interactions and activity of microbes in their living environment. The diversity of microbial communities in deep terrestrial subsurface varies from single or few-species ecosystems to communities with significant diversity (Tyson et al. 2004, Chivian et al. 2008, Wouters et al. 2013, Nyyssönen et al. 2014). The diversity of a microbial community is measured by the number of unique operational taxonomic units (OTUs) corresponding to a kind of organisms or species, found in individual samples (Sogin et al. 2006). This is called the α-diversity. One commonly used diversity measure is the Shannon index (H'), describing both richness and evenness of the taxa in the community. Richness is the number of species or different taxa in the sample. Evenness de-

scribes how close are the total numbers of different species in the environment. In addition to richness and evenness estimates, rarefaction analyses also describe the community's diversity, particularly how well the diversity of each habitat has been captured (Sogin et al. 2006). Multivariate statistics measurements such as analysis of principal coordinates are used to characterize the β-diversity, i.e. the diversity between different communities and abiotic factors affecting the microbial community structures (Griffiths et al. 2011, Barberán et al. 2012).

The β-diversity links to biogeography, i.e. a concept of placing microbes on the map (Martiny et al. 2006). It is stated that the global spatial distribution of free-living microbes depends on the environmental factors determining the selection pressures. Biogeography of deep-dwelling microbial lineages has been under study recently in hydrothermal vent systems (Anderson et al. 2015). The origin of microbes in deep subsurface is also linked to biogeography. Microbes colonizing the deep subsurface are most likely transferred from surface environments concurrently with slow movement of water or have been trapped and isolated between depositional events (Amy et al. 1992). For example in Äspö, Sweden, and in Olkiluoto, Finland, deep aquifers contain groundwater mixed from different sources, such as glacial melt, old Litorina Sea and recent Baltic Sea in addition to some very old saline water (Laaksoharju et al. 1999, Posiva 2012).

Changing gears from global to minute scales, it is likely that microbial processes in deep subsurface occur in spatially isolated microenvironments. This is true for example in soil aggregates (Mummey et al. 2006). Such microenvironments are often formed on interfaces of solid material and water, forming a miniature microbial ecosystem called a biofilm. Biofilms form also in deep subsurface (Anderson et al. 2006, Wanger et al. 2006, MacLean et al. 2007). However, these are mostly monolayers or irregular patches of exopolysaccharides and sporadically distributed microbes (Anderson et al. 2006, Wanger et al. 2006). Consequently, most of the microbial ecology studies of the deep subsurface to date have concentrated on planktonic communities.

1.5.1 Culture-dependent methods

Microbial ecology has been studied traditionally with cultivation–based methods, such as plate counting and most probable number methods. These have also been used to describe the microbial communities in deep subsurface environments (Pedersen et al. 2008, Hallbeck and Pedersen 2008a, 2012). The advantage of these methods is that once a certain microbial strain is purified and growing in the laboratory conditions, the physiology of the strain can be characterized, the strain can be taxonomically classified and material for further studies can be continuously collected. However, the major setback is that only 0.01–0.1% of the total microbial cells in the aquatic environments are cultivable or produce visible colonies with standard plating methods (Pedersen et al. 2008). However, with careful development of the methods for example for anaerobic groundwater, much higher yields in microbial numbers can be obtained (Pedersen et al. 2008).

1.5.2 Marker genes as molecular biological tools

Identification of microbes

The 16S subunit of ribosomal RNA gene, i.e. 16S rRNA gene provides the basis for culture-independent microbial diversity investigations. The 16S rRNA gene has been the golden standard for defining evolutionary relationships between microbes since Woese et al. divided life on Earth into the three domains; *Bacteria*, *Archaea* and *Eucarya* (Woese 1987, Woese et al. 1990). The 16S rRNA gene occurs in all prokaryotic life forms and it has a relatively slow mutation rate. Due to these features, it is a good molecular chronometer that allows determination of phylogenetic relationships of microbes and identification based on comparison to databases. The sequence information stored in several databases is growing rapidly as high-throughput sequencing and metagenomic analyses combined with bioinformatics tools are providing increasing amount of novel information (e.g. Rinke et al. 2013, Petitjean et al. 2015). For example, Ribosomal Database Project's (RDP) current release 11.4 from May 2015 consists of over 3.2 million aligned and annotated 16S rRNA sequences[1].

Targeting microbes with specific functionality

Since the demonstration of congruence of phylogenetic trees of bacterial 16S rRNA and the gene coding for the ATP-synthase (Amann et al. 1988), a range of functional genes have been used as (phylogenetic) markers for metabolic processes, many of these in deep marine subsurface (Blazejak and Schippers 2011, Lever et al. 2013, Lever 2013, Nercessian et al. 2005, Newberry et al. 2004). An ideal functional marker gene must fulfill certain requirements (Lever 2013). First of all, the enzyme that the marker gene encodes is only used for one specific reaction. A database containing enough gene sequences has to be available for the identification of the functional gene sequences acquired from environmental samples. The marker gene must be conserved so that general or group-specific molecular probes can be designed to match the gene sequence. Lastly, the evolutionary history of the gene must be known and possible lateral gene transfer should be recognized.

The contribution of deep subsurface microbial communities to the global elemental cycling has been studied with a variety of functional marker gene assays (Lever 2013). The most studied functions are sulfate reduction and methanogenesis (Teske and Biddle 2008). The dissimilatory sulfite reductase (*dsrAB*) and adenosine 5′-phosphosulfate reductase (*aprA*) genes have been used to widely describe the sulfate reducing microbial communities in the deep biosphere (e.g. Baker et al. 2003, Blazejak and Schippers 2011, Bomberg et al. 2015a, Itävaara et al. 2011a,b, Lever et al. 2013, Meyer and Kuever 2007, Moser et al. 2005, Nakagawa et al. 2002, Nyyssönen et al. 2012, Tiago and Veríssimo 2013). Methano-

[1] http://rdp.cme.msu.edu/misc/rel10info.jsp

gens have been detected using the methyl coenzyme M reductase gene (*mcrA*) (e.g. Bomberg et al. 2015a, Fry et al. 2009, Dhillon et al. 2005, Moser et al. 2005, Nyyssönen et al. 2012). However, many functional genes used as marker genes do not completely meet these characteristics. For example, the dissimilatory sulfite reductase enzyme, coded by the gene *dsrAB* is also operating in the opposite direction, i.e. for sulfide oxidation (Loy et al. 2009). However, these functions can be separated because sulfate reducers generally fall to different genetic clusters than the sulfide oxidizers. Similarly, the methyl coenzyme M reductase coded by the *mcrA* gene may work in both directions depending on whether it is used by anaerobic methane oxidizers or methanogens (Hallam et al. 2004). However, thus far the *mcrA* of methanogenic archaea and anaerobic methanotrophic archaea (ANME) fall into different phylogenetic branches so that the function of the methyl coenzyme M reductase can be deduced from the nearest neighbors in the phylogenetic tree (Knittel and Boetius 2009).

Other functional genes involved in carbon cycling pathways such as aerobic methanotrophy and methylotrophy, acetogenesis and autotrophic carbon fixation have been analyzed from deep subsurface environments (e.g. Lever et al. 2010, Rajala et al. 2015, Tiago and Veríssimo 2013). Marker genes used for the detection of methanotrophy and methylotrophy are the particulate methane monooxygenase gene (*pmoA*) and the methanol dehydrogenase gene (*mxaF*), respectively. Lever et al. (2010) used the formyl tetrahydrofolate synthetase gene (*fsh*) as a functional marker gene for acetogenesis in deep subseafloor sediments. The ribulose-1,5-bisphosphate carboxylase/oxygenase, shortly RuBisCO enzyme is involved in the autotrophic carbon fixation via the Calvin-Bassham-Benson cycle. Genes coding for two forms of this enzyme (*cbbL* and *cbbM*) have been used for characterization of chemolithoautotrophic organisms in deep hydrothermal vents (Campbell and Cary 2004, Takai et al. 2005). Takai et al. (2005) also used genes coding key enzymes of another type of autotrophic carbon fixation pathway, the reductive tricarboxylic acid cycle (ATP citrate lyase beta subunit gene *aclB*, pyruvate:ferredoxin oxidoreductase gene *porAB*, 2-oxoglutarate: ferredoxin oxidoreductase gene *oorAB*). In addition to these genes, Tiago and Veríssimo (2013) also used the *accC* gene coding the acetyl coenzyme A carboxylase:biotin carboxylase, in order to reveal the dominant carbon fixation pathway in a deep subsurface aquifer. The key microbial players in nitrogen cycling in the deep biosphere have been identified with functional genes for nitrogen fixation (H-subunit of nitrogenase gene complex, *nifH*) and nitrate reduction (nitrate reductase gene *narG*) (Lau et al. 2014, Orsi et al. 2013, Rajala et al. 2015).

1.6 Deep biosphere studies

Although some natural environments and pre-existing manmade infrastructure can allow sampling from the deep biosphere, in many cases drilling technologies are required to reach greater depths (Sahl et al. 2010, Moser et al. 2003, Onstott et al. 2009, Wilkins et al. 2014). Even though the drilling operations are expensive and

rigorous, there are several locations around the globe where such operations have been and currently are conducted (Wilkins et al. 2014, ICDP web page[2]). The deep terrestrial biosphere has been studied in many of these sites (**Table 1**). These locations differ greatly in lithology, age, geochemical composition and microbiology. The deep biosphere of the Kalahari Shield in South Africa and the Canadian Shield in North America represent the most ancient environments mainly composed of metamorphosed sedimentary rocks (e.g. Stotler et al. 2009, Onstott et al. 2006). The formation of the Fennoscandian Shield also dates to the Precambrian and the dominant rock types in deep bedrock of the Fennocandian Shield are granites and high metamorphic grade gneisses (e.g. Haveman et al. 1999, Haveman and Pedersen 2002a, Pedersen et al. 2008, Itävaara et al. 2011b). The youngest rocks hosting a deep terrestrial biosphere are reported from Japan (Fukuda et al. 2010, Shimizu et al. 2006, Mills et al. 2010). The temperature range spans over sixty degrees of °C and pH varies from mildly acidic to very alkaline (eg. Sahl et al. 2008, Stevens 1993). Most saline fluids are detected from Driefontein (total dissolved solids, (TDS) 103 g l^{-1}, Onstott et al. 2006, Katz et al. 2011), Olkiluoto (TDS 125 g l^{-1}, Ahokas et al. 2014) and Outokumpu (TDS 68.9 g l^{-1}, Kietäväinen et al. 2013). Microbial presence in deep terrestrial subsurface has been confirmed in many locations around Earth, which are discussed in the next chapters. In fact, only few studies, where microbial presence has been searched for, have been unsuccessful in detecting prokaryotic life in deep subsurface. However, the very low cell counts in deep subsurface and/or the inability to obtain enough representative sample material might have affected to these results (Colwell and D'Hondt 2013).

Studies show that deep terrestrial subsurface environments host microbial communities with structural similarities. These include the frequent detection and domination of the microbial communities by Proteobacteria and Firmicutes (e.g. Zhang et al. 2006, Gihring et al. 2006, Lin et al. 2006a,b, Shimizu et al. 2006, Fukuda et al. 2010, Rastogi et al. 2010, Itävaara et al. 2011a, b, Nyyssönen et al. 2014, Articles I and III). These bacterial phyla also frequently dominate the microbial communities of deep subsurface rock samples (Zhang et al. 2005, Sahl et al. 2008). Sulfate reducing microbes and methanogens have been discovered from several deep subsurface sites around the globe (**Table 1**). Sulfate reduction is a significant energy-yielding process in many of these sites and the numbers of sulfate reducers can be high (Moser et al. 2005, Stotler et al. 2011, Hallbeck and Pedersen 2012). On the other hand, the abundance of methanogens in the microbial communities in deep terrestrial subsurface environments is often low (e.g. Fry et al. 1997, Hallbeck and Pedersen 2012, Nyyssönen et al. 2014). Nevertheless, methanogen community composition appears to be depth-related in bedrock, as many aceticlastic methanogens are relatively more abundant in shallower depths, whereas autotrophic methanogens are more frequent in deeper fluids (Kietäväinen and Purkamo 2015).

[2] http://www.icdp-online.org/projects/

Table 1. Characteristics of several deep terrestrial subsurface sites where microbes have been detected.

Site		Main rock types	Age (rock)[1] Ma	Depth[1] mbs	T[1] °C	pH	Salinity[1] TDS g L^{-1}	DIC mM	TOC mM	DOC mM	Detected microbes[4]	References[5]
Canada	Lupin	Metagraywacke, slate, banded iron formation	2600	1130	11	7.9 … 9.2	40	0.088 … 0.20	0.12 … 0.33	0.15 … 0.36	B, SRB	37, 53, 54
China	Donghai	Granitic gneiss, paragneiss, eclogite, garnet peridotite	240	3350	87	9.2 … 9.4					A, B	57
Finland	Hästholmen	Rapakivi granite	1630	985		6.8 … 8.5	31.8	0.38 … 2.22	0.30 … 0.34	0.08 … 0.82	SRB, IRB, AG	15, 16, 27
	Kivetty	Granite, granodiorite	1880	855		7.8 … 9.0	0.23	0.99 … 2.15	0.18	0.14 … 1.27	SRB, IRB, AG	3, 15, 16, 43
	Olkiluoto	Migmatitic gneiss, mica gneiss, granite	1850	960	19	7.8 … 8.3	125	0.04 … 5.9	0.12 … 0.14		SRB, IRB, AG, MG, MT, ANME	1, 5, 15, 16, 34, 41
	Outokumpu	Mica schist, black schist, granodiorite, serpentinite	1900	2480	40	8.4 … 10.1	68.9				SRB, MG, MT	17, 22, 35, 44, 45, 46, 47
	Palmottu	Garnet-cordierite gneiss, granite	1900	417		6.2 … 9.4	1.6	0.5 … 3[2]			SRB, IRB, AG	2, 18, 40
	Romuvaara	Tonalite gneiss	2700	566		8.4	0.17	1.85	1.05		SRB, IRB, AG	15, 16, 42
Japan	Horonobe	Mudstones, sandstone	23	625	36	6.8		450		31	SRB, MG	51
	Mizunami	Granite	75	1169	22	8.2 … 8.6		0.12 … 0.18		0.10 … 0.13	B	10
	Tono	Granite	68		25	7 … 10		0.08 … 4		170 … 1190	MT	28
	Toyoha	Pyrite, chalcopyrite		550	71						A, SRB	32
South Afric	Beatrix	Conglomerate, quartzite	2900	1390	40	7.7 … 7.9		0.34	0.14 … 0.18	0.14	B, MG	6, 11, 25, 56
	Driefontein	Andesite, quartzite	2700	3300	43	6.0 … 7.4	103	0.41 … 0.57	0.40 … 1.83	0.67	SRB, MG, ANME	4, 6, 11, 20, 25, 30, 31, 50
	Evander	Conglomerate, quartzite	2900	2230	45	7.2 … 8.6			0.22 … 0.61		SRB, MG, ANME	8, 11, 25, 56
	Kloof	Andesite	2700	3400	59	7.6 … 8.5	16.5	0.02[3]	0.46 … 1.67		A, B	11, 20, 21, 25, 50, 56
	Mponeng	Metabasalt	2700	3300	52	9.3	11.8		0.43		B, MG	11, 20, 25, 26, 50
Sweden	Forsmark	Meta-granite	1900	1002		7.3 … 8.3	15	0.04 … 1.5[2]		0.12 … 0.13	IRB, SRB, AG, MG, MT	6, 12, 14
	Laxemar	Granite, quartz monzodiorite	1800	922	19	7.5 … 8.4	18	0.13 … 5.4[2]	0.12 … 1.67	0.12 … 1.75	IRB, SRB, AG, MG	12, 13, 14, 39
	Äspö	Granite, granodiorite	1800	860	19	6.8 … 7.8	16	0.16 … 0.34[2]	0.11 … 0.57	0.025 … 1.5	IRB, SRB,N, AG, MG, MT	7, 12, 19, 23, 24, 39
USA	Columbia River Basalt	Basalt	23	1270	18	9.9	0.7	1.07 ... 2.84		0.12 … 0.44	IRB, SRB, MG	3, 9, 52
	Henderson mine	Granite	2500	1044	40	5.8 … 6.3		15.7 … 32		0.03 … 0.05	N, A	49, 55
	Homestake, SURF	Metasediments	1900	1478	33	6.6 … 8.5					SRB, IRB, N, MG, MT	29, 38, 48
	Snake River Plain	Basalt	10	235	15	7 … 8.3				0.08 … 0.22	A, MT	33, 36

[1]Maximum values are given for age, depth, temperature (T) and salinity

[2]HCO_3^- concentration

[3]TIC

[4]A=archaea, B=bacteria, SRB=sulphate-reducing bacteria, IRB=iron-reducing bacteria, N=N-cycling microbes, AG=acetogens MG = methanogens, MT= aerobic methanotrophs, ANME = anaerobic methanotrophs

[5]1) Ahokas et al. (2014), 2) Ahonen et al. (2004), 3) Anttila et al. (1999), 4) Baker et al. (2003), 5) Bomberg et al. (2015a), 6) Borgonie et al. (2011), 7) Chi Fru (2008), 8) Davidson et al. (2011), 9) Fry et al. (1997), 10) Fukuda et al. (2010), 11) Gihring et al. (2006), 12) Hallbeck and Pedersen (2008a), 13) Hallbeck and Pedersen (2008b), 14) Hallbeck and Pedersen (2012), 15) Haveman et al. (1999), 16) Havemann and Pedersen (2002b), 17) Itävaara et al. (2011a), 18) Kaija et al. (1998), 19) Kalyuzhnaya et al. (1999), 20) Katz et al. 2011 21) Kieft et al. (2005), 22) Kietäväinen et al. (2013), 23) Kotelnikova and Pedersen (1997), 24) Kotelnikova and Pedersen (1998), 25) Lin et al. (2005a), 26) Lin et al. (2006b), 27) Luukkonen et al. (1999), 28) Mills et al. (2010), 29) Morelli et al. (2010) and references therein, 30) Moser et al. (2003), 31) Moser et al. (2005), 32) Nakagawa et al. (2002), 33) Newby et al. (2004), 34) Nyyssönen et al. (2012), 35) Nyyssönen et al. (2014), 36) O'Connell et al. (2003), 37) Onstott et al. (2009), 38) Osburn et al. (2014), 39) Pedersen and Ekendahl (1990), 40) Pedersen and Haveman (1999), 41) Pitkänen and Partamies (2007), 42) Pitkänen et al. (1996), 43) Pitkänen et al. (1998), 44) Purkamo et al. (2013)(ArticleI), 45) Purkamo et al. (2015)(ArticleII), 46) Purkamo et al. (2015)(ArticleIII), 47) Rajala et al. (2015), 48) Rastogi et al. (2009), 49) Sahl et al. (2008), 50) SherwoodLollar et al. (2006), 51) Shimizu et al. (2006), 52) Stevens (1993), 53) Stotler et al. (2009), 54) Stotler et al. (2012), 55) Swanner et al. (2011), 56) Ward et al. (2004), 57) Zhang et al. (2006)

1.6.1 South Africa

Gold mines in South Africa have provided relatively easy access to the deep subsurface. Thus, microbial ecology of the deep biosphere of Kalahari Shield has been extensively characterized during the last 15 years (e.g. Takai et al. 2001, Gihring et al. 2006, Silver et al. 2010, Davidson et al. 2011, Lau et al. 2014) (**Table 1**). The diversity of microbial communities was reported to be low likely due to the scarcity of nutrients and electron acceptors in the borehole and bedrock fracture fluids (Gihring et al. 2006). However, many species of SRB and methanogenic archaea, in addition to an archaeal candidate division (SAGMEG) without any cultured representatives to date have been discovered from the deep biosphere of Witwatersrand Basin (Takai et al. 2001, Moser et al. 2003, 2005, Lin et al. 2006a, Gihring et al. 2006, Blanco et al. 2014). Furthermore, some intriguing findings from these deep bedrock sites have been made. For example, a unique single-species ecosystem was discovered from a bedrock fracture zone from Mponeng mine. Candidatus *Desulforudis audaxviator* as a sole member of the fracture fluid community is an apparent self-sufficient organism capable of surviving in this environment over a long period of time by using geologically produced sulfate and hydrogen (Chivian et al. 2008). In addition, Borgonie et al. (2011, 2015) found nematodes in the deep bedrock fracture fluids and from the stalactites growing from the ceilings of the mines. One entirely new species of nematode, *Halicephalobus mephisto* was described from the Beatrix mine from a depth of 1300 m below ground level (Borgonie et al. 2011).

1.6.2 Asian locations

Among the deepest drilling projects where microbial ecology has been studied is the Chinese Continental Scientific Drilling Project in Donghai, China. The drill hole is located in the Dabie-Sulu ultra-high pressure metamorphic belt, formed during the Triassic, in a convergent plate boundary in Eastern China. Microbial communities were characterized from rock cores and drilling fluids from a depth range of 0.5–3.3 km (Zhang et al. 2005, 2006). Proteobacteria dominated the clone libraries derived from the rock cores. α-proteobacterial sequences were only derived from the rock sample from the shallowest depth at 529 m, while β-proteobacteria were prominent in the rock core from 730 m depth. Furthermore, *Pseudomonas*-related clone sequences became more abundant with depth (Zhang et al. 2005). Similar proteobacteria also dominated the drilling fluids above 2 km, but below this depth Firmicutes predominated in Dabie-Sulu (Zhang et al. 2006).

In Japan, deep terrestrial biosphere studies have been linked to reliable and safe geological disposal of nuclear waste. Two different sites, Mizunami Underground Research Laboratory[3] and Horonobe Underground Research Center[4] have been engaged in this research (Niibori 2015). Mizunami, located in central Japan

[3] http://www.jaea.go.jp/04/tono/miu_e/index.html
[4] http://www.jaea.go.jp/english/04/horonobe/

offers access to Cretaceous granitic crystalline bedrock groundwater, where β-proteobacteria dominated the microbial community at 1.1 km depth (Fukuda et al. 2010). In Horonobe, northern Japan, on the other hand, several experimental boreholes have been drilled into the Wakkanai formation. This formation is comprised of sandstones with high porosity but low permeability and is divided by a major fault zone. The Omagari fault divides the area to a northeastern and a southwestern side, and the microbial communities at these sites differed significantly from each other (Shimizu et al. 2006). β-proteobacteria dominated the microbial community and methanogens were not detected in the northeastern side of the fault at the depth of 458 m. At the southwestern side of the fault zone at 374 m depth, a possible sulfate-methane transition zone (SMTZ) with sulfate reducing Firmicutes and δ-proteobacteria living together with methanogenic archaea was described. Interestingly, the archaeal sequences were all affiliating closely with a single archaeal species, *Methanoculleus shikugoensis* (Shimizu et al. 2006).

Near Mizunami lies Tono uranium mine, where Mills et al. (2010) detected two different microbial ecosystems from a borehole intersecting Toki lignite-bearing and granite formations at depths of 160-200 m. Microbial cell membrane phospholipid fatty acid (PLFA) signatures suggested that the microbial community in lignite-bearing formation could utilize ancient recalcitrant organic matter originating from lignite. It was hypothesized that fermentative heterotrophs may be responsible for the initial breakdown of this material, possibly in a syntrophic relationship with methanogens. The granitic bedrock on the contrary hosted chemolithoautotrophic microbes using dissolved inorganic carbon and possibly H_2 produced in radiolysis of water in the uranium rich rocks of the formation for primary production. In addition, specific signatures of aerobic methanotrophs were detected from these samples (Mills et al. 2010).

1.6.3 North America

The deep biosphere of the Canadian Shield has been mostly studied with emphasis on hydrogeochemistry (e.g. Sherwood Lollar et al. 1993, 2006, Stotler et al. 2012, Holland et al. 2013). However, microbial communities and microbial sulfate reduction activity have been detected from groundwater samples from the continuous permafrost area of the Lupin gold mine and High Lake, respectively (Onstott et al. 2009, Stotler et al. 2011). The studies demonstrated that sulfate reduction was the dominant microbial process in these environments reaching depths over 500 m. Recently, Holland et al. (2013) reported record-breaking 1.5 Ga residence times of fracture fluids from Timmins mine in Canadian Precambrian Shield.

In USA, deep biosphere has also been studied through mines. Sahl et al. (2008) described the microbial communities of deep fractures and a rock core sample from Precambrian granitic bedrock of the Henderson molybdenum mine. Fluid samples taken from two different packer-isolated fractures represented similar microbial communities, dominated by bacterial phylotypes closely affiliating to each other. These phylotypes formed an intrinsic candidate division Henderson Group 1 that had no close phylogenetic relationship with any described bacterial

species. However, these microbes might play a role in nitrogen fixation in deep biosphere in the Henderson mine deep subsurface (Swanner and Templeton 2011). The rock core microbial community was dominated by the β-proteobacterial family *Ralstoniaceae* and Firmicutes.

After the closure of the mining activities at Homestake gold mine in 2001, The Sanford Underground Research Laboratory (SURF) was established to provide scientific opportunities for direct exploration of the deep subsurface in this deepest mine in North America reaching the depth of 2.4 km. Microbial ecology of the mine was studied by Rastogi et al. (2009, 2010) and Osburn et al. (2014). Proteobacteria dominated both the clone libraries and the PhyloChip biosignatures from soil samples and high-throughput sequences from borehole fluids (Rastogi et al. 2009, 2010, Osburn et al. 2014). Many of the proteobacterial species reported from Homestake mine have been previously detected from gold mines in Japan and South Africa (Rastogi et al. 2010).

In situ -evidence from Columbia River Basalts (CRB) supports the hypothesis of hydrogen-driven deep biosphere (Gold 1992, Stevens and McKinley 1995, Pedersen 1997) to some extent. Stevens et al. (1993) reported that viable bacterial populations reflect the groundwater chemistry of Grande Ronde and Priest Rapids aquifers in Columbia River Basalts at depths of 1270 and 316 m, respectively. Subsequently, Stevens and McKinley (1995) provided evidence for the existence of subsurface lithotrophic microbial ecosystem (SLiME) in CRB. Enrichment of the heavier stable carbon isotope, ^{13}C, below 200 m depth suggests preferential removal of ^{12}C by methanogens. However, bacterial species, including active hydrogen-oxidizing sulfate reducers and homoacetogens are more abundant than methanogens that comprise only a small fraction of the total microbial community in CRB (Fry et al. 1997).

Even though methanogenic Archaea have been suggested to be the primary producers in photosynthesis-independent deep terrestrial biosphere (Gold 1992, Pedersen 1997), as in CRB and other sites, the numbers of methanogens are low. However, Chapelle et al. (2002) described a subsurface microbial community composed of over 90% methanogens relying on hydrogen and CO_2 from Lidy Hot Springs, Idaho. This community differed from all other previously described deep terrestrial subsurface microbial ecosystems and filled most of the criteria proposed to be designated as a true SLiME, i.e. that chemolithotrophic organisms are present and metabolically active using solely geological energy source and electron acceptors in this ecosystem (Nealson et al. 2005).

1.6.4 Fennoscandia

Microbial ecology of Precambrian Fennoscandian Shield has been studied for more than two decades (e.g. Pedersen and Ekendahl 1990, 1992, Pedersen 1997, Kotelnikova and Pedersen 1998, Haveman et al. 1999, Haveman and Pedersen 2002, Hallbeck and Pedersen 2008a,b, 2012, Itävaara et al. 2011a,b, Nyyssönen et al. 2012, Pedersen 2012a,b, Nyyssönen et al. 2014, Bomberg et al. 2014, 2015a,b, Articles I–III). The most extensive studies on microbial ecology in granitic

bedrock have probably been done in Äspö Hard Rock Laboratory (HRL)[5] that has been running since 1995. Other important study sites include planned nuclear waste repository sites of Olkiluoto in Finland and Laxemar-Simpevarp and Forsmark in Sweden. This thesis is based on studies conducted in Outokumpu Deep Scientific Drill Hole in Finland (see the following section).

Microbial communities have been characterized by resolving the dominant taxa and enumeration of different physiological groups from both Finnish and Swedish sites (Pedersen et al. 1996, Haveman et al. 1999, Haveman and Pedersen 2002, Pedersen et al. 2008, Hallbeck and Pedersen 2008a,b, 2012, Nyyssönen et al. 2012) (**Table 1**). These studies have relied on traditional most probable number (MPN) cultivation methods and molecular biological methods, such as clone library sequencing and community fingerprinting methods. Metabolically diverse communities comprised of autotrophic and heterotrophic acetogens, nitrate, iron, manganese and sulfate reducing microbes and methanogens have been identified in these studies. Interestingly, the microbial communities differ from site to site and from one drill hole to another (Haveman and Pedersen 2002, Pedersen et al. 2008, Hallbeck and Pedersen 2008a, 2012, Nyyssönen et al. 2012, Itävaara et al. 2011a,b). Nevertheless, these studies conclusively propose that nitrate reduction, sulfate reduction and acetogenesis are significant energy-yielding processes for deep subsurface microbes at these sites. In addition, aerobic methane-oxidizing microbes have been detected from Äspö, Forsmark and Olkiluoto fluids from shallow depths (Pedersen et al. 2008, Chi Fru 2008). In Outokumpu, evidence for aerobic methane oxidation capacity has been demonstrated in the 500 m fracture (Rajala et al. 2015). Methanogens on the other hand are present especially at greater depths in these environments, representing only a minority of the total microbial community (Pedersen et al. 2008, Hallbeck and Pedersen 2012, Nyyssönen et al. 2012). High-throughput sequencing analyses have revealed more precisely the microbial community composition at different depths of Olkiluoto deep biosphere. β-, γ- and δ-proteobacteria are the most dominant classes whereas α- and ε-proteobacteria represent lower relative abundance in various fractures at depths above 200 m and below 385 m (Bomberg et al. 2014). A sulfate-methane transition zone lies in between these depths in Olkiluoto, and at these depths, ε-proteobacteria are dominating the communities (Bomberg et al. 2015a). This zone hosts typical indicator organisms for anaerobic methane oxidization, such as ANME-1 archaea in addition to ANME-2D archaea (Pedersen 2013, Bomberg et al. 2015a). Thus, the detection of ANME archaea supports the previously proposed possibility of anaerobic methane oxidation in Olkiluoto deep bedrock based on the detection of *mcr*A genes affiliating with those of ANME-1 archaea (Nyyssönen et al. 2012). In addition to ANME archaea, the other dominating archaeal groups in Olkiluoto fractures are *Methanobacteriales* and *Thermoplasmatales* -related representatives (Bomberg et al. 2014, Bomberg et al. 2015a). In addition to prokaryotic life, fungal communities have been characterized from Äspö and Olkiluoto sites (Pedersen et al. 1996, Sohlberg et al. 2015).

[5] http://www.skb.com/research-and-technology/laboratories/the-aspo-hard-rock-laboratory/

1.7 The Outokumpu Deep Drill Hole

This thesis is based on studies executed in the Outokumpu Deep Scientific Drill Hole, located in Eastern Finland (62.72°N, 29.07°E). The vertical drill hole reaches the depth of 2516 m in Palaeoproterozoic crystalline bedrock in Fennoscandian Shield (**Figure 2**). The 22 cm in diameter drill hole is cased only to the depth of 39 m, thus providing access to the crystalline bedrock *in situ* below this depth. The Outokumpu district is characterized as a classical ore province in Finland with its copper, cobalt and zinc sulfide deposits. Drilling was conducted during the years 2004–2005 by a Russian company NEDRA in collaboration with The Geological Survey of Finland (Kukkonen 2011). The core drilling technique used steel-tooth drilling bits, and municipal tap water was used as the primary drilling fluid. Drilling fluid conditioners were used to stabilize the drill hole wall only when necessary, mostly above 1000 m. The drilling fluid was labeled with fluorescein dye to estimate the mixing of the intrinsic formation water and drilling fluid. When drilling was finished, the drill hole was flushed with fresh tap water. Gradual replacement and mixing of this fresh water with saline formation fluids in the drill hole has been observed over several years of hydrogeochemical monitoring in the Outokumpu site (Ahonen et al. 2011, Kietäväinen et al. 2013).

The drill core reveals a cross section of the lithology of the Outokumpu bedrock, comprising of metasedimentary, igneous and ophiolite-related rocks, such as mica schist, pegmatitic granite and serpentinite and skarn, respectively. Numerous thin black schist veins are detected throughout the drill hole lithology (Västi 2011). The groundwater in the area is typical for shield brines affected by long-term water-rock interactions. Sodium, calcium and chloride ions build up salinity in Outokumpu groundwater. In addition to characteristic salinity, fluids contain ample amount of dissolved gas of which CH_4 is the major component above 2000 m (Kietäväinen et al. 2013). Five different water types have been detected in Outokumpu Deep Drill Hole, based on the geochemistry and isotopic composition of the water stable isotopes, each emanating from different fracture zone of the bedrock (Kietäväinen et al. 2013). According to the noble gas stable isotope composition and concentration, the age of the formation fluids is estimated to be at least several tens of millions of years (Kietäväinen et al. 2014).

The low-porosity bedrock of continental crust in Outokumpu provides a unique environment to study photosynthesis-independent, nutrient-poor but still very alive deep biosphere. The microbial communities in the drill hole water column of Outokumpu bedrock have previously been characterized (Itävaara et al. 2011a,b, Nyyssönen et al. 2014), and recently Rajala et al. (2015) described methanotrophic subpopulations from a fracture fluid at 500 m depth. Proteobacteria and Clostridia dominated in the drill hole water column, although the bacterial community structure varied at different depths (Itävaara et al. 2011a,b, Nyyssönen et al. 2014). At 200 and 600 m depths, *Comamonadaceae* and *Acholeplasmataceae* were the most abundant phyla, while the highest diversity was detected between depths from 1000–1500 m. Here, the proportion of clostridial phylotypes was exceptionally high compared to other depths (Nyyssönen et al. 2014). *Methanobac-*

terium has been shown to be the most common archaeal genus throughout the drill hole water column (Itävaara et al. 2011a, Nyssönen et al. 2014) with the exception of the depth 1100 m, where *Methanolobus* dominated (Nyyssönen et al. 2014). Methanogens represent only a fraction of the total microbial community in the Outokumpu deep biosphere determined by the low copy numbers of the methanogenesis marker gene, *mcrA*, compared to the total amount of microbes (Itävaara et al. 2011a). Metagenomic analysis of microbial communities at 600, 1500 and 2300 m depths of the drill hole revealed metabolic potential for different types of autotrophic carbon fixation, but also for fermentation of organic acids at 1500 m, possibly due to the high proportion on fermentative clostridia detected at this depth (Nyyssönen et al. 2014). Although these microbial communities in Outokumpu are probably fairly inactive, some microbial groups can readily respond to environmental changes. This was demonstrated in a recent study, in which the transcription of marker genes for methane oxidation, sulfate reduction and nitrate reduction was rapidly stimulated when the microbial communities derived from the 500 m fracture in Outokumpu were supplied with methane and SO_4^{2-} (Rajala et al. 2015).

However, the intrinsic microbial communities of the fractures from where the ancient fluids are emanating to the Outokumpu drill hole have not been characterized. In addition, only a little is known about the functionality of these microbial communities, and even less about the connections between these microbes and the ecosystem functions.

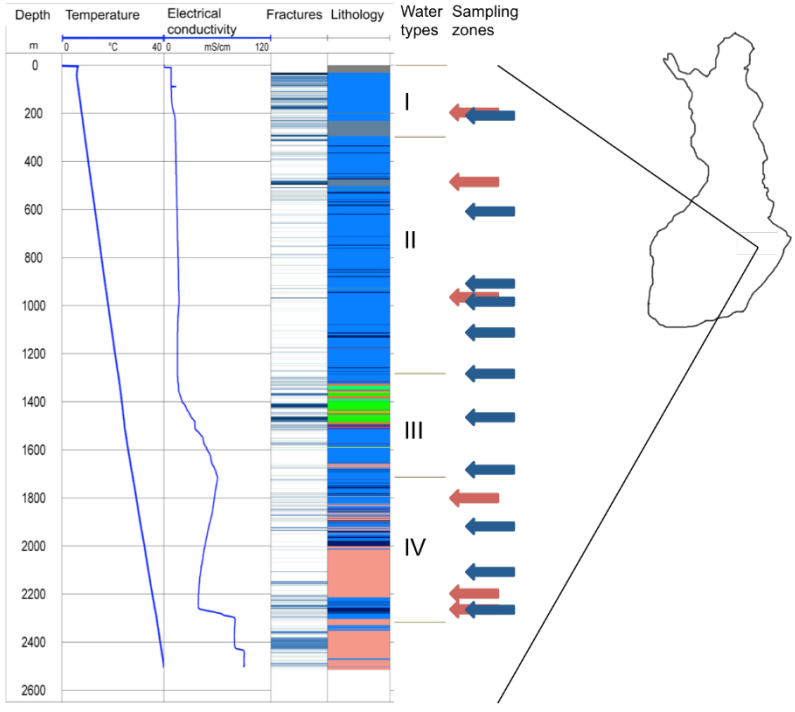

Figure 2. Schematic of in situ temperature, electrical conductivity, fracturing and lithology, water types and sampling depths of the bedrock in Outokumpu Deep Drill Hole in Finland (Kukkonen et al. 2011a,b, Västi 2011, Kietäväinen et al. 2013). Fractures are indicated with dark lines. Lithology is described as follows: grey and blue indicate metasediments, green and orange indicate ophiolite-derived rock types and pink pegmatitic granite. Water types are marked with roman numerals, studied fracture depths are indicated with red arrows and studied depths of the drill hole water column with blue arrows. (Modified from Article I.)

2. Aims of this thesis

The objective of this work was to characterize the microbial communities living in the deep Fennoscandian bedrock biosphere in Outokumpu, in addition to estimate the functionality of the archaeal and bacterial communities in this habitat. Moreover, this work targeted on identification of the core microbial community and the key players of the communities in addition to the description of species interactions.

Modern molecular biological methods based on the genetic properties of microbes were used to resolve the structure and the metabolic properties of microbial communities. This work concentrates on the intrinsic microbial communities dwelling in the pristine fractures of the deep crystalline bedrock in addition to the functionality and carbon cycling in the water column of the Outokumpu Deep Scientific Drill Hole.

- Developing methods for microbiological sampling of the Outokumpu bedrock fractures and assessing the feasibility of these sampling methods for collection of indigenous fracture fluids from deep crystalline bedrock (Article I). Hypothesis: the fracture zones in Outokumpu host endemic microbial communities that differ from the drill hole water column communities. The sampling method must be verified in order to ensure that the samples are derived from the actual fracture fluids.
- Characterization of the microbial communities present and functioning in the Outokumpu deep subsurface (Article I and III). Hypothesis: Diverse microbial communities are detected from Outokumpu deep crystalline bedrock biosphere. Microbial communities are similar to those found in the deep subsurface in other Fennoscandian Shield sites.
- Characterization and enumeration of microbes with key metabolic properties important in risk assessment of industrial utilization of deep subsurface (sulfate reduction and methanogenesis) (Article I, II and III). Hypothesis: Sulfate reduction and methanogenesis are significant functions in the anaerobic deep subsurface.
- Determination of the dominant carbon fixation metabolism of microbial communities of Outokumpu Deep Drill Hole (Article II and III). Hypothesis: Autotrophy is the dominant type of metabolism, as organic carbon is scarce in Outokumpu deep bedrock.

- Discovering the links between the microbial community composition, functionality of the community and the geochemical factors in deep crystalline bedrock biosphere (Article III). Hypothesis: The chemical properties of the fracture fluids and the prevailing lithology at each fracture shape the microbial community structure, for example by determining the electron acceptors, carbon and/or nutrient sources or favoring specialized extremophiles.

3. Materials and methods

This chapter shortly describes the materials and methods used in this study. More detailed information and references are presented in the original publications and in **Table 2**.

3.1 Sampling of the fracture fluids and drill hole water column

Fracture fluid samples were collected from fracture zones located at six different depths (**Figure 2**). Two techniques were applied for collection of intrinsic fracture fluids: I) pumping the fluid from the fracture isolated from the rest of the drill hole water with the expandable rubber and stainless steel packers (Lapela Oy, Finland) placed above and below each zone (Ahonen et al. 2011) (**Figure 3**) and II) a slow continuous pumping for several weeks from the level of each fracture zone allowing the fluids to discharge from the fracture (Article I, Article III). The fluid was pumped through a sterile PA tube initially filled with mQ or RO water. In both cases stable levels of pH, electrical conductivity and oxygen detected with continuous monitoring during the pumping ensured that indigenous water was obtained.

Drill hole water column was sampled using a 2300 m long sterile polyamide tube with 50 m long sections connected together by sterilized tube fittings or ball valves and a backpressure valve at the lower end (Nurmi and Kukkonen 1986). The tube was lowered into the drill hole and allowed to slowly fill up with drill hole fluid. The tube was then lifted whereupon the backpressure valve closed, and each ball valve was closed immediately as it emerged from the drill hole. Each 2 × 50 m section of the tube was treated as one sample representing a 100-m interval of the drill hole (Nyyssönen et al. 2014). Microbiological analyses were made from 11 depths ranging from 200–2300 m (**Figure 2**.) (Article II.)

3.2 Hydrogeochemical measurements

Electrical conductivity, pH, redox potential, temperature, and oxygen levels were continuously monitored and recorded during the pumping of the fractures. Cation and anion composition, alkalinity and gas composition of the fluids were analyzed

as described in Ahonen et al. (2011), Nyyssönen et al. (2014) and in Article I. Water for cation analysis was filtered and acidified with ultrapure HNO_3. Analysis was done with ICP-MS or ICP-OES. Anions were determined with ion chromatography. Alkalinity was titrimetrically measured, and gas composition was analyzed with gas chromatography with TCD and FID detectors. Due to the high salinity in the drill hole, analysis of many chemical components of the fluid was problematic.

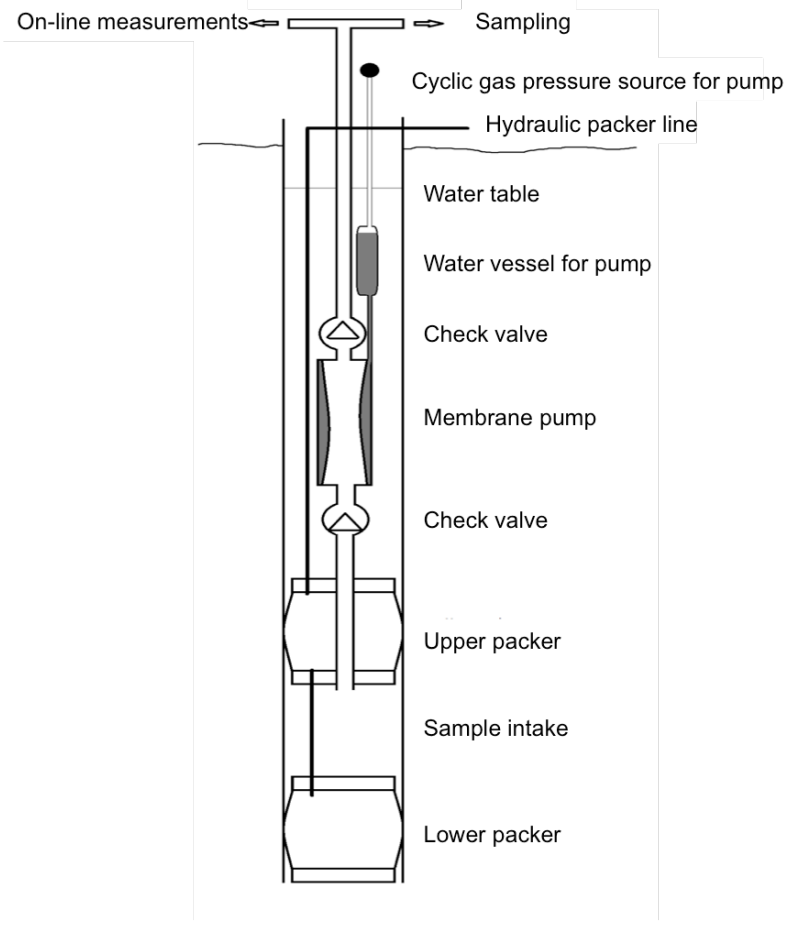

Figure 3. Schematic drawing of the packer system used in Outokumpu (courtesy of L. Ahonen).

3.3 Enumeration of microbes

Enumeration of microbial cells was conducted with microscopy or by molecular biological methods. Samples for microscopy were placed into acid-washed, sterile headspace glass flasks in the anaerobic chamber and closed with butyl rubber stoppers and aluminum crimp caps. Samples were kept chilled prior to staining with fluorescence stains (Article I, II, III, Itävaara et al. 2011b, Nyyssönen et al. 2014). Either Live/Dead or DAPI fluorescent stains were used to visualize the cells in the samples. The number of viable and dead cells was determined with the BacLight Bacterial Viability Kit (Molecular Probes, Invitrogen Corp., USA) (Article I, II) as described in Itävaara et al. (2011b). DAPI-staining was conducted as described in Article I.

Quantitative PCR (qPCR) was used in enumeration of the copy numbers of several marker genes in the samples. The copy number of 16S rRNA gene was used as an approximation of bacterial and archaeal cell numbers in fractures (Article III). The copy numbers were determined from DNA with qPCR targeted either to bacterial or archaeal 16S rRNA gene using Roche LightCycler technology and commercial qPCR mastermix (**Table 2**) as described in Article III.

The abundance of functional marker genes used as a proxy for key anaerobic respiration processes, sulfate reduction and methanogenesis was calculated with qPCR. Amplification protocols are described in detail in Articles I and III, commercial kits and used primers are shown in **Table 2** and **Table 3**. The numbers of SRB and methanogens were estimated by comparing the amplification result to a standard dilution of *Desulfobulbus propionicus* DSM 2554 *dsrB* gene and *Methanothermobacter thermoautotrophicus* DSM 1053 *mcrA* gene, respectively.

Table 2. Microbiological and molecular biological methods used in this thesis.

Assay	Method	Target gene[1]	Details[2]	Article	Reference[3]
Cell density	DAPI staining			I,III	2
Cell wall integrity, amount of living vs. dead cells	Live/Dead staining		LIVE/DEAD Bacterial Viability Kit (BacLight), Molecular Probes, Invitrogen	I,III	3
DNA extraction	Bead beating		PowerSoil DNA isolation kit, MoBio	I, II, III	
RNA extraction	Bead beating		PowerWater RNA isolation kit, MoBio	I, II, III	
Purification of RNA	DNase treatment		RQ1 Rnase-Free Dnase, Promega	I,III	
Production of cDNA from RNA	reverse transcription		SuperscriptIII, Invitrogen	I, III	
Increasing DNA yield	WGA		Illustra GenomiPhi V2, GE Healthcare	II	
Bacterial community fingerprint analysis	DGGE	16S rRNA		I	5
Bacterial community identification	HTP sequencing	16S rRNA		III	4
Archaeal community fingerprint analysis	DGGE	16S rRNA		I	5
Archaeal community identification	HTP sequencing	16S rRNA		III	4
Sulphate reducing community fingerprint analysis	DGGE	*dsrB*		I, II	5
Sufate reducing community identification	HTP sequencing	*dsrB*		III	4
Methanogen community analysis	Cloning	*mcrA*	TOPO TA Cloning Kit, Invitrogen	II	6
Methanogen community identification	HTP sequencing	*mcrA*		III	4
Carbon assimilation via chemoorganotrophy	DGGE	*accC*		II	6
Carbon assimilation via Calvin cycle	Cloning	*rbcL*, *cbbM*	TOPO TA Cloning Kit, Invitrogen	II	6
Carbon assimilation via Wood-Ljungdahl pathway	Cloning	*acsB*	TOPO TA Cloning Kit, Invitrogen	II	6
Methanotrophic community analysis	Cloning	*pmoA*	TOPO TA Cloning Kit, Invitrogen	II	6
Nitrate reducing community analysis	Cloning	*narG*	TOPO TA Cloning Kit, Invitrogen	II	6
Ammonia-oxidizing community fingerprint analysis	DGGE	*amoA*		II	5
Quantification of bacterial 16S rRNA gene copy number	qPCR	16S rRNA	KAPA SYBR® Fast 2× Master mix, Kapa Biosystems, Roche LightCycler 480	III	1
Quantification of archaeal 16S rRNA gene copy number	qPCR	16S rRNA	KAPA SYBR® Fast 2× Master mix, Kapa Biosystems, Roche LightCycler 480	III	1
Quantification of methyl-coenzyme M reductase gene copy number	qPCR	*mcrA*	KAPA SYBR® Fast 2× Master mix, Kapa Biosystems, Roche LightCycler 480	III	1
Quantification of dissimilatory sulphate reduction gene copy number	qPCR	*dsrB*	KAPA SYBR® Fast 2× Master mix, Kapa Biosystems, Roche LightCycler 480	I,III	1

[1]see details: Table 3

[2]used commercial kits

[3]of the original method, 1) Higuchi et al. (1993), 2) Kepner and Pratt (1994), 3) Lloyd and Hayes (1995), 4) Margulies et al. (2005), 5) Muyzer et al. (1993), 6) Pace et al. (1986)

3.4 Biomass collection, nucleic acids extraction and subsequent analyses

Biomass from the fracture fluids was collected in the field laboratory in a portable anaerobic chamber using filtration (Article I, III). Sampling fluid was pumped directly into the anaerobic chamber via the sampling tube and biomass was collected from a defined amount of sample fluid (1 L or 500 mL) on sterile cellulose acetate filters of 0.2 µm pore size (**Figure 4**). Biomass was stored at -80 °C prior to DNA and RNA extraction.

Figure 4. Biomass filtration in the anaerobic cabinet of the field laboratory.

Biomass from the drill hole fluids was collected from 11 depths. Sampling was performed directly from the tube section through a flame-sterilized, pressure-tight valve. Water samples for nucleic acid analyses (500 ml each) were filtered directly with Sterivex filter units and immediately frozen on dry ice. Prior to DNA extraction, filters were cut from the filter units and sliced into smaller pieces before placing them on the extraction kit's bead tube.

DNA and RNA were extracted from the biomass with commercial extraction kits (**Table 2**) according to manufacturer's instructions. RNA was transcribed to cDNA as described in Articles I and III, whole genome amplification of drill hole water samples was done according to manufacturer's instructions and described in Article II and in Nyyssönen et al. (2014). The PCR amplification procedures for subsequent molecular biological analyses of fracture and drill hole fluid samples were conducted as described in Articles I–III.

3.5 Microbial community characterization

Microbial community studies were conducted either by denaturing gradient gel electrophoresis (DGGE) (Article I) or high-throughput sequencing methods (Article III). DGGE and cloning in addition to Sanger sequencing were applied for functional marker gene studies (Article II). PCR primer details used in molecular biological characterization and assessment of functionality of the microbial communities are presented in **Table 3**.

DGGE profiling of bacterial, archaeal, SRB and dark carbon fixating communities

PCR products containing a GC clamp at the 5′-end of the forward primer were run on denaturing gradient gel electrophoresis (DGGE) with gene-specific gradient, voltage and run time described in Articles I (16S rRNA gene of bacteria and archaea, *dsrB*) and II (*accC*, *dsrB*).

Prominent bands were excised from the DGGE gels and DNA from the gel fragment was allowed to suspend to 20 µl of molecular-grade water overnight at +4 °C. DNA from each band was reamplified using the same PCR protocols as in original PCR and sequenced at Macrogen, Inc. (South Korea).

Cloning of marker genes for methanogenesis, methanotrophy and nitrate reduction

Amplified PCR products were purified from agarose gel slices using commercial gel extraction kits and ligated with a plasmid vector overnight at +12 °C. Transformation reaction was performed according to the manufacturer's instructions using chemically competent *Escherichia coli* cells. The transformants were grown on Luria-Bertani agar plates containing kanamycin as a selective agent at 37 °C overnight. Clones were checked for insert with colony PCR. PCR products of clones containing an insert of the expected size as determined by agarose gel electrophoresis were sequenced at Macrogen, Inc., Korea. Detailed information and reaction conditions are described in Article II.

High throughput sequencing of bacterial, archaeal, SRB and methanogenic communities

Barcoded primers were used to produce amplicon libraries of bacterial and archaeal 16S rRNA, *dsrB* and *mcrA* genes. The composition of the reaction mixes and run conditions are described in Article III and the used primers in **Table 3**. PCR products were verified with agarose gel electrophoresis and successful reactions from replicate reactions were pooled prior to sequencing. The sequencing was performed at the Research and Testing Laboratory, Texas, USA (180 m sam-

ples) and at the Institute of Biotechnology, Helsinki, Finland (all other samples) using the 454 FLX Titanium platform.

Statistical methods

DGGE gel images were normalized, Dice's coefficient of similarity was calculated and UPGMA cluster dendrograms were constructed in order to compare the similarity of the DGGE fingerprint profiles using the Bionumerics software. (Articles I and II).

The PAST software (Hammer et al. 2001) was used to test the normality and perform a canonical correspondence analysis on presence/absense data of phylotypes of different functional genes in connection to geochemistry (Article II) or microbial community structure and geochemical variables of the fractures (Article III).

Table 3. Primers used in molecular biological characterization of Outokumpu deep biosphere.

Target gene	Gene name	Method	Primer names forward / reverse	Forward primer sequence 5'->3'	Reverse primer sequence 5'->3'	Size of the product,	Article	Reference[*]
Ribosomal gene coding the 16S subunit of rRNA in bacteria	16S rRNA	PCR-DGGE	P2 / P3	ATTACCGCGGCTGCTGG	CCTACGGGAGGCAGCAG[1]	193	I,III	11
		qPCR	P1 / P2	CCTACGGGAGGCAGCAG	ATTACCGCGGCTGCTGG	193	I,III	11
		HTP sequencing	8f / P2	AGAGTTTGATCCTGGCTCAG	ATTACCGCGGCTGCTGG	500	I, II, III	4, 11
Ribosomal gene coding the 16S subunit of rRNA in archaea	16S rRNA	PCR-DGGE	A344f / 519rP	ACGGGGCGCAGCAGGCGCGA	TTACCGCGGCKGCTG[2]		I, II, III	2, 18
		nested PCR 1. step	A109f / A915r	ACKGCTCAGTAACACGT	GTGCTCCCCCGCCAATTCCT	800	I,III	7, 16
		qPCR	A109f / A744r	ACKGCTCAGTAACACGT	CCCGGGTATCTAATCC		I, III	7, 10
		HTP sequencing	A344f / A744r	ACGGGGCGCAGCAGGCGCGA	CCCGGGTATCTAATCC	430	II	2, 10
Dissimilatory sulfite reductase gene, β-subunit	*dsrB*	PCR-DGGE	Dsr2060f+GC / Dsr4r	CAACATCGTYCAYACCCAGGG	GTGTAGCAGTTACCGCA[1]	370	III	6, 17
		qPCR	Dsr2060f / Dsr4f	CAACATCGTYCAYACCCAGGG	GTGTAGCAGTTACCGCA		I	6, 17
		HTP sequencing	Dsr2060f / Dsr4f	CAACATCGTYCAYACCCAGGG	GTGTAGCAGTTACCGCA	370	III	6, 17
Methyl coenzyme M reductase, α-subunit	*mcrA*	PCR-cloning	M1 / M2	GCMATGCARATHGGWATGTC	TCATKGCRTAGTTDGGRTAGT	760	I, II	8
		nested PCR 1. step for cloning	mcrA412f / mcr1615r	GAAGTHACHCCNGAAACVATCA	GGTGDCCNACGTTCATBGC		III	14
		qPCR	M1 / M3	GCMATGCARATHGGWATGTC	TGTGTGAAWCCKACDCCACC		II	8, 14
		nested PCR 1. step for HTP-seq	mcrA463f / mcrA1614r	GAAGTHACHCCNGAAACVATCA	GGTGDCCNACGTTCATBGC	1200	III	14
		HTP sequencing	M1 / M3	GCMATGCARATHGGWATGTC	TGTGTGAAWCCKACDCCACC		III	8, 14
Acetyl coenzyme A carboxylase, biotin carboxylase subunit	*accC*	PCR-DGGE	ACAC2254f-GC / ACAC720r	GCTGATGCTATACATCCWGGWTAYGG	GCTGGAGATGGAGCYTCYTCWATTA	460	II	1
Acetyl coenzyme A synthase, β-subunit	*acsB*	PCR-cloning	ACS_f / ACR_r	CTBTGYGGDGCIGTIWSMTGG	AARCAWCCRCADGADGTCATIGG	216	II	5
Ribulose-1,5-bisphosphate carboxylase/oxygenase, large subunit	*rbcL*	PCR-cloning	K2f / V2r	ACCAYCAAGGCCSAAGCTSGG	GCCTTCSAGCTTGCCSACCRC	490	II	12
Ribulose-1,5-bisphosphate carboxylase/oxygenase, form II	*cbbM*	PCR-cloning	RuIIF1 / RuIIR3	GGHAACAACCARGGYATGGGYGA	CGHAGIGCGTTCATGCCRCC	800	II	15
		PCR-cloning	RuIIF2 / RuIIR2	GGIACVATCATCAARCCVAA	TGRCCIGCICGRTGRTARTGCA	488	II	15
Particulate methane monooxygenase, α-subunit	*pmoA*	PCR-cloning	pmo1f / pmor	GGGGGAACTTCTGGGGITGGAC	GGGGGRCIACGTCITTACCGAA	330	III	3
Nitrate reductase, α-subunit	*narG*	PCR-cloning	1960m2f / 2050m2r	TAYGTSGGGCAGGARAAACTG	CGTAGAAGAAGCTGGTGCTGTT	110	III	9
Ammonia monooxidase, α-subunit	*amoA*	PCR-DGGE	amoA1f / amoA-2R+GC	GGGGTTTCTACTGGTGGT	CCCCTCGGCAAAGCCTTCTTC[3]	490	III	13

[*]GC-clamp:CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGG

[1]GC-clamp:CGCCCGCCGCGCCCGCGCCGTCCCGCCGCCCCCGCCC

[2]GC-clamp:CGCCGCGCGGCGGGCGGGGCGGGGGC

[*]1) Auguet et al. (2008), 2) Bano et al. (2004), 3) Cheng et al. (1999), 4) Edwards et al. (1989), 5) Gagen et al. (2010), 6) Geets et al. (2006), 7) Großkopf et al. (1998), 8) Hales et al. (1996), 9) López-Gutiérrez et al. (2004), 10) modified from Barns et al. (1994), 11) Muyzer et al. (1993), 12) Namba et al. (2004), 13) Nicolaisen and Ramsing (2002), 14) Nyyssönen et al. (2012), 15) Spiridonova et al. (2004), 16) Stahl and Amann (1991), 17) Wagner et al. (1998), 18) Øvreås et al. (1997)

Sequence analyses

Sequences from DGGE and clone libraries were manually checked, edited, aligned and phylogenetic trees were constructed with the Geneious Pro software (Article I and II). In addition, the functional gene sequences acquired were compared to previously published shotgun-sequenced metagenomic libraries from Outokumpu (Article II, Nyyssönen et al. 2014) using the Blast algorithm in Geneious Pro.

High-throughput sequenced amplicon libraries were analysed using mothur and QIIME programs (Schloss et al. 2009, Caporaso et al. 2010). An in-house developed QIIME-based pipeline was used with 16S rRNA gene sequences and mothur with the functional gene sequences. The setup for quality control for each gene is described in detail in Article III. 16S rRNA gene sequences were compared against Greengenes representative OTU set version gg_13_8 with 97% similarity and the taxonomy was assigned according to RDP (Wang et al. 2007). Functional gene sequences aligned with model alignments of *dsrB* and *mcrA* obtained from Fungene repository (Fish et al. 2013). Final taxonomy of the representative OTUs was obtained by comparing sequences to public sequence databases using Geneious Pro. α-diversity estimates (Shannon H', Chao1, ACE) were calculated in QIIME for 16S rRNA gene sequence data, using 97% species similarity from datasets normalized with random subsampling of sequences according to the sample with lowest number of sequence reads (bacteria 3030, archaea 270)(Article III).

All sequences retrieved from DGGE, clone libraries and HTP sequencing libraries were deposited in the European Nucleotide Archive[6], and the accession numbers are presented in **Table 4**.

Table 4. Accession numbers of the sequences retrieved in this study.

Gene	Method	Accession numbers	Article
bacterial 16S rRNA	DGGE	HF565417-HF565444	I
	HTP-sequencing	ERS846377-ERS846388	III
archaeal 16S rRNA	DGGE	HF565395-HF565416	I
	HTP-sequencing	ERS846389-ERS846397	III
*acc*C	DGGE	HG967562-HG967579	II
*dsr*B	DGGE	HF565370-HF565394	I
		HG967613-HG967637	II
	HTP-sequencing	ERS846399-ERS846407	III
*mcr*A	cloning	HG967593- HG967612	II
	HTP-sequencing	ERS846408-ERS846414	III
*nar*G	cloning	LN589977-LN589983	II
*pmo*A	cloning	HG967580-HG96759	II

[6] http://www.ebi.ac.uk/ena

3.6 Prediction of functionality and co-occurrence analysis

Article III describes in detail the estimation of the functional content of predicted metagenomes of Outokumpu microbial communities. Metagenomes were reconstructed from the 16S rRNA and rRNA gene sequence data with the PICRUSt program (Langille et al. 2013). OTUs without taxonomic reference were removed from the taxonomy data, which was subsequently uploaded to Galaxy pipeline (Goecks et al. 2010, Blankenberg et al. 2010, Giardine et al. 2005) for PICRUSt. Weighted nearest sequenced taxon indexes (NSTI) were calculated and metagenomes predicted from the normalized taxonomy data. Normalization was done by dividing the abundance of each organism by its predicted 16S rRNA gene copy number because of the variation between the copy numbers of 16S rRNA gene in different microbes. From the predicted metagenomes, the presence/absence and abundance of selected metabolic pathways of microbes was determined computationally using the HUMAnN program (Abubucker et al. 2012).

Correlation coefficients for OTUs present in the microbial communities were calculated with the otu.association command in mothur. The co-occurrence based on significant ($p < 0.01$) pairwise Pearson correlations between different OTUs was visualized with biological network analysis program Gephi (Bastian et al. 2009). With the network analysis the keystone genera and the connectivity of the microbial communities in the deep Outokumpu bedrock were determined.

4. Results

4.1 Obtaining the samples from the Outokumpu deep biosphere

During the years when hydrogeochemistry and microbiology have been under study in Outokumpu, besides the continuous development of the sampling techniques and equipment, also field instrumentation and working methods have been refined.

In 2008, water samples were collected from the drill hole using the tube sampling method. Altogether 11 samples were recovered from the drill hole water column for microbial analyses and 19 for chemical analyses (depths 200–2300 m). Generally, the bacterial communities at different depths of the drill hole resembled one another, although the relative abundance of the different groups varied between the depths. The most dominant phylotypes belonged to *Comamonadaceae*, *Acholeplasma* and *Clostridiales*. The archaeal communities changed towards higher abundance of *Methanobacter* in greater depths (Nyyssönen et al. 2014).

In 2009 isolation of the fracture zones with packers and continuous pumping methods were taken to use. These methods enabled the collection of greater amount of samples and obtaining the biomass in anaerobic conditions in the field laboratory. In order to ascertain that samples were attained from the indigenous fracture fluids, electrical conductivity, pH and concentration of oxygen of the water were continuously monitored throughout the time of pumping. After installation of the sampling gear and start of the pumping, water chemistry rapidly stabilized to that of the in-situ conditions regardless of the sampling method (either packer isolation or slow flow through PA tube, see Section 3.1). The microbial cell numbers decreased during the pumping period. At the end of the pumping period, the highest numbers of microbial cells were detected in shallow fractures and the lowest from the deeper fractures, thus the cell number was decreasing according to the depth. In addition, changes in microbial community structure were detected during the pumping, especially in fractures located deeper, where the microbial diversity decreased according to DGGE analysis (Article I). The bacterial communities of the fracture zones shared similarity with the drill hole communities especially in the beginning of the pumping. β-proteobacterial phylotypes dominated the microbial communities in 500 m and 967 m fracture zones after one hour of pump-

ing, as was the case with the bacterial community in 600 m depth in the drill hole. During the pumping, clostridial phylotypes became more dominant in microbial community at the 967 m fracture, therefore resembling more the communities in the drill hole water column at depths of 1100–1500 m. The bacterial community at 2260 m fracture with the dominant phylotype belonging to actinobacteria differed from the drill hole communities approximately at the same depth already in the beginning of the pumping. However, the bacterial communities of the fracture zones at these depths differ also from one another and characterization of the bacterial community structure in 2260 m with HTP sequencing verified the results of the community analysis made with DGGE (Article III).

The archaeal community structure changed less than the bacterial community during the pumping, and communities resembled the drill hole communities especially in 1000–2300 m depths. SAGMEG archaea dominated the community in the 967 m fracture of the fracture zone and were abundant also in the archaeal communities in the drill hole at depth range of 200–1000 m.

4.2 Microbial community structure

The microbial communities derived from RNA were used as a proxy of an active community and the data acquired from DNA as a representation of the total community present in the samples. Overall, the bacterial communities detected in the fractures were more diverse than the archaeal communities (Articles I and III). All studied fractures differed in their bacterial community composition, although in the fractures from shallower depths, the total and active bacterial communities did not differ as much as they did in the deeper ones. Most diverse total bacterial communities were detected in the fractures at 1820 and 2300 m depth (H' = 6.3) (**Table 5**a). β-proteobacterial *Comamonadaceae* were the dominant representatives of the bacterial communities at 180 and 500 m fractures (**Figure 5**). The DGGE- and pyrosequencing results were compatible with each other concerning the bacterial communities at 500 m fracture (**Table 6**) (Articles I and III). In the 967 m fracture, clostridial phylotypes *Dethiobacter* and *Syntrophobotulus* dominated the total and active communities, respectively. In the 1820 m fracture, the total bacterial community was characterized by high diversity (H' = 6.3), with γ-proteobacterium *Pseudomonas* being the most abundant genus (15% relative abundance). In contrast, Firmicutes dominated the active community with relative abundance of over half of the community. Noticeably less proteobacterial phylotypes were observed in the active community than in the total community in the 1820 m fracture (6% relative abundance). It was observed with both DGGE and HTP sequencing that actinobacteria dominated the total bacterial community in the 2260 m fracture (**Table 6**). Candidate phylum OPB41 comprised approximately half of the total community and *Comamonadaceae* was the second most abundant group (33%) in the 2260 m fracture. The active community differed significantly from the total community in this fracture, while most abundant group was α-proteobacterial *Bradyrhizobium* (20%). The majority of the phylotypes of the total community in

the 2300 m fracture belonged to β-proteobacterial *Burkholderiales* (*Comamonadaceae*, *Leptothrix*, *Janthinobacterium*) and γ-proteobacterial *Pseudomonas* and *Acinetobacter*. The active community of this fracture represented mostly unknown phylotypes that could be characterized only to kingdom or phylum level.

Table 5. HTP-sequence diversity, abundance and richness estimates of a) bacteria and b) archaea. The data were normalized with random subsampling according to the sample with lowest number of sequence reads in each dataset.

A) BACTERIA

	180 m		500 m		967 m		1820 m		2260 m		2300 m	
Diversity index	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA
Shannon	2,9	2,6	3,4	4,9	5,3	5,1	6,3	3,5	2,7	5,1	6,3	1,8
Chao1	208	125	428	461	326	363	523	538	394	492	495	209
ACE	230	144	463	483	340	388	546	558	430	531	529	229
observed species	143	90	329	395	274	318	432	412	286	446	424	116

B) ARCHAEA

	180 m		500 m		967 m		1820 m		2260 m		2300 m	
Diversity index	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA
Shannon	2,4	2,1	0,9	0,8	2,3	1,2	n.d	n.d	0,6	0,8	0,8	n.d
Chao1	45	32	3	3	58	32	n.d	n.d	7	8	5	n.d
observed species	44	29	3	3	58	30	n.d	n.d	7	8	4	n.d

n.d = not detected

Table 6. Most abundant bacterial phyla detected from the Outokumpu fracture communities with two different molecular biological methods.

Fracture depth	DGGE		HTP-sequencing	
m	DNA	RNA	DNA	RNA
180	n.a	n.a	*Comamonadaceae*	*Comamonadaceae*
500	*Rhodobacteraceae*	*Rhodobacteraceae*	*Comamonadaceae*	*Comamonadaceae*
	Comamonas	*Comamonas*	*Rhodobacter*	*Rhodobacter*
	Acholeplasma	*Acholeplasma*	*Acholoeplasma*	*Dietzia*
967	*Peptococcaceae*	*Peptococcaceae*	*Comamonadaceae*	*Syntrophobotulus*
	Uncl. Firmicutes	Uncl. Firmicutes	*Dethiobacter*	*Clostridia*
	Acholeplasma	*Acholeplasma*	*Acholeplasma*	
1820	n.a	n.a	*Pseudomonas*	Uncl. Bacteria
			Uncl. Firmicutes	Uncl. Firmicutes
			Dethiosulfatibacter	*Dethiosulfatibacter*
2260	*Arthrobacter*	*Dethiosulfatibacter*	OPB41 (Actinobacteria)	*Micrococcus*
			Comamonadaceae	*Bradyrhizobium*
				Uncl. Firmicutes
				Uncl. Bacteria
2300	n.a	n.a	Uncl. Firmicutes	Uncl. Firmicutes
			Comamonadaceae	Uncl. Bacteria
			Pseudomonas	
			Acinetobacter	

n.a = not analyzed

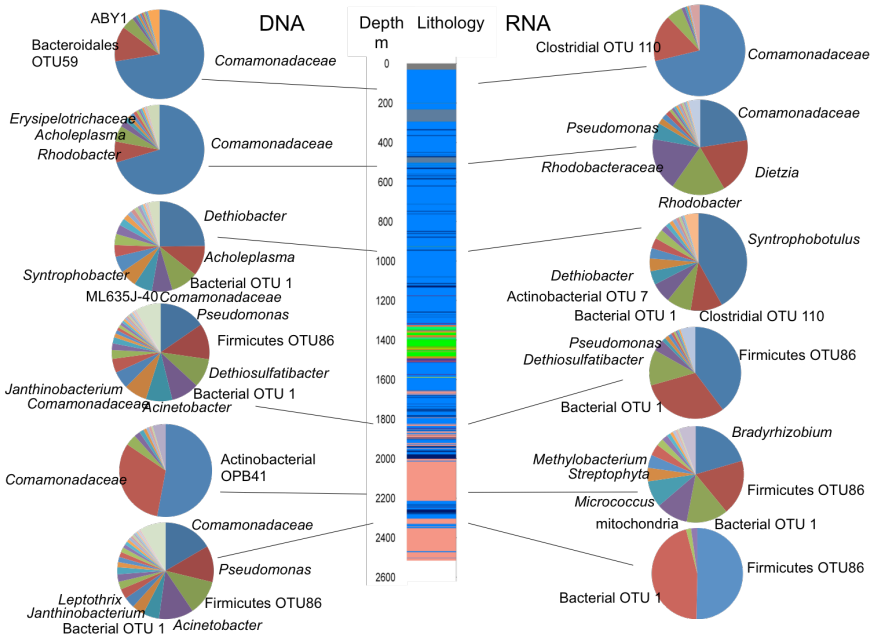

Figure 5. The bacterial community structure of the studied Outokumpu bedrock fractures at different depths. The bacterial groups with the highest relative abundance are named. The structures for the total communities are shown on the left and the active communities on the right of the Outokumpu lithology map in the middle. Explanation of the lithology can be found from **Figure 2**. The black line indicates the depth from which each sample originates (adapted from Article III).

The archaeal community structure revealed with DGGE matched with that obtained with the HTP sequencing (**Figure 6** and **Table 7**). *Methanobacteraceae*-like hydrogenotrophic methanogens dominated all archaeal communities except that of the fracture at 967 m depth. The total community of this fracture was dominated by archaea affiliating with candidate phylum SAGMEG-1. The active archaeal community at 967 m resembled the ones in the other fractures with *Methanobacteriaceae* being the most abundant group. The most diverse archaeal community was found in the fracture zone at 180 m (H' = 2.4), in which *Methanobacteraceae* dominated but also hydrogenotrophic *Candidatus Methanoregula* and methylotrophic *Methanolobus* archaea were abundant. The archaeal community at 967 m fracture had the highest amount of observed archaeal OTUs (58) (**Table 5**b).

Table 7. Most abundant archaeal phyla detected from the Outokumpu fractures with two different molecular biological methods.

Fracture depth	DGGE		HTP-sequencing	
m	DNA	RNA	DNA	RNA
180	n.a	n.a	*Methanobacteriaceae* Candid. *Methanoregula*	*Methanobacteriaceae* Candid. *Methanoregula* *Methanolobus*
500	*Methanobacteriaceae*	*Methanobacteriaceae*	*Methanobacteriaceae*	*Methanobacteriaceae*
967	SAGMEG-1	SAGMEG-1	SAGMEG-1	*Methanobacteriaceae*
1820	n.a	n.a	n.a	n.a
2260	*Methanobacteriaceae*	*Methanobacteriaceae*	*Methanobacteriaceae*	*Methanobacteriaceae*
2300	n.a	n.a	*Methanobacteriaceae*	*Methanobacteriaceae*

n.a = not analyzed

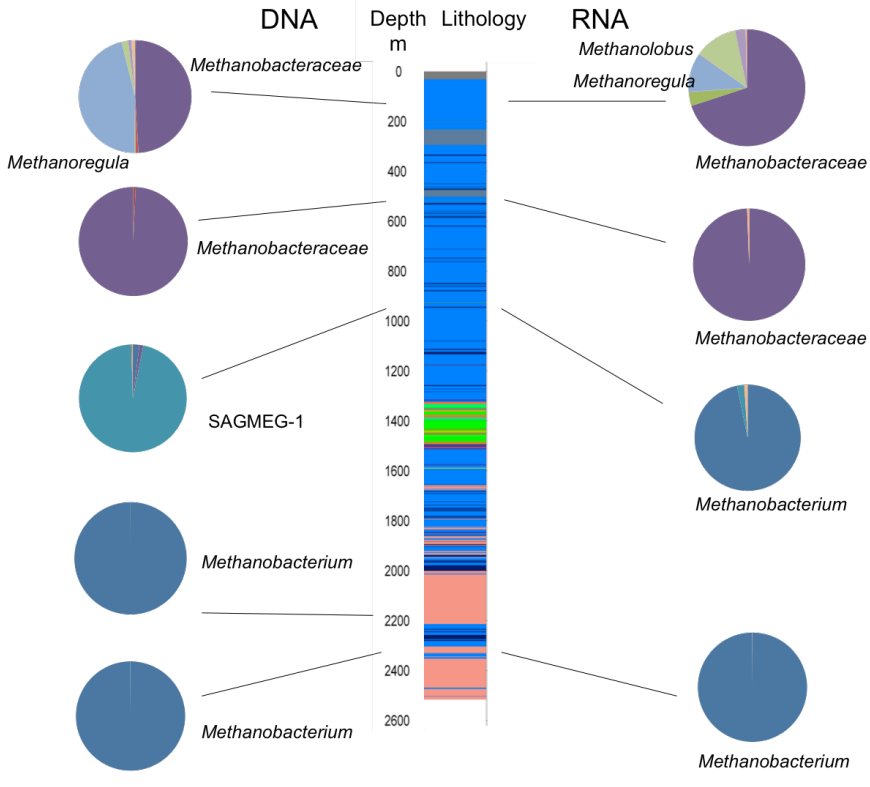

Figure 6. The archaeal community structure of the studied Outokumpu bedrock fractures at different depths. The phylotypes with the highest relative abundance are named. The structures for the total communities are shown on the left and the

active communities on the right of the Outokumpu lithology map in the middle. Explanation of the lithology can be found from **Figure 2**. The black line indicates the depth from which each sample originates (adapted from Article III).

4.3 The core microbial community

A phylotype is considered to belong to the core microbiome of Outokumpu deep biosphere if it is detected in all communities of Outokumpu fractures. Only a few proteobacterial and clostridial phylotypes constituted the core microbiome in Outokumpu deep bedrock (Article III) (**Table 8**). These phylotypes were detected in all fracture communities at different frequencies. When the total and active communities were observed separately, the core community of the total bacterial communities was composed of 14 phylotypes, of which *Comamonadaceae* were most abundant in addition to *Dethiobacter* and *Pseudomonas*. In contrast, only four phylotypes were detected in all active bacterial communities (**Table 8**). In addition, *Methanobacteraceae* -affiliating methanogenic archaea could be regarded as part of the core microbiome in Outokumpu, as *Methanobacterium* was detected in the archaeal communities at fracture depths of 967 m and below and *Methanobacteraceae* dominated the communities in 180 m and 500 m fractures. Many members of the core microbiome in Outokumpu deep subsurface represented only a minority in the communities.

Table 8. The core bacterial communities of the Outokumpu deep bedrock fractures.

	range of the relative abundance (%)			
	in the total community		community	
	min	max	min	max
Comamonadaceae	7	72		
Dethiobacter	0.04	25		
Pseudomonas	0.02	15	0.06	6
Firmicutes OTU86	0.1	12	0.03	50
Bacterial OTU2	1	10	1	46
Dethiosulfatibacter	0.05	10	0.07	12
Actinobacterial OTU7	0.09	4		
Leptothrix	0.2	4		
Legionella	0.07	4		
Geosporobacter	0.1	2		
Clostridia OTU110	0.01	1		
Burkholderiales OTU197	0.02	0.7		
Clostridiales OTU111	0.01	0.5		
Peptococcaceae	0.02	0.3		

4.4 Carbon cycling in the Outokumpu deep biosphere

Carbon assimilation

Three autotrophic carbon assimilation pathways, the reductive pentose phosphate cycle (Calvin cycle), the reductive acetyl-CoA pathway (Wood-Ljungdahl pathway) and the hydroxypropionate-hydroxybutyrate pathway were tested with marker gene assays to investigate whether carbon assimilation in Outokumpu deep biosphere occurs via an autotrophic pathway or are organic substrates a more prominent source of carbon. The acetyl-coA carboxylase:biotin carboxylase (*accC*) gene fragments used as a marker for the hydroxypropionate-hydroxybutyrate pathway were detected from all studied depths (200–2300 m). (**Table 9**.) *AccC* gene fingerprints were divided to four clusters corresponding to the depth from which they were retrieved (Article II). *Parabacteroides* and novel Outo II group *accC* sequences were detected in upper parts of the drill hole, while clostridial and *Methanobacterium* -type *accC* were detected below 1100 m. Neither of the common autotrophic carbon fixation pathways (Calvin-Bassham-Benson or Wood-Ljungdahl cycle) was detected from Outokumpu drill hole fluids (Article II). In addition, analysis of the predicted metagenomes showed that the relative abundance of the genes involved in these autotrophic carbon fixation pathways were low in the fracture communities (Article III).

Methanogenesis and methanotrophy

While copy numbers of the marker gene for methanogenesis, methyl-coenzyme M reductase (*mcrA*), were extremely low or below the detection limit throughout the drill hole and fracture fluids, many archaeal phylotypes detected in the drill hole samples and from the fractures were characteristically methanogens. Cloning of the *mcrA* gene revealed a distinct spatial distribution pattern. At shallower depths in the drill hole, *Methanosarcina* -affiliating *mcrA* sequences were more abundant, while *Methanolobus* -types of *mcrA* sequences were found at 1300 and 1500 m depths and *Methanobacterium* were exclusively found from the deepest samples at 1900 m and below. Methanogen communities of the fracture fluids were comparable to those in the drill hole fluid at approximately corresponding depths, with *Methanobacteriaceae* dominating the total communities in the fractures located at 2260 m and 2300 m depths (**Table 9**). *Methanosarcina* were abundant in the active methanogen communities in the 500 and 967 m fractures as in the drill hole fluids at depths of 600 and 900 m (Articles II and III). The detection of *Methanolobus mcrA* sequences at 1300–1500 m depths is in agreement with the archaeal community structure at these depths, where *Methanolobus* was present with approximately 10–15% relative abundance (Article II and Nyyssönen et al. 2014).

Methanotrophs were detected at 1500 m and above from the drill hole. All sequenced *pmoA* clones were similar to each other and affiliated with *Methylomonas pmoA* sequences (Article II) (**Table 9**).

Table 9. Comparison of microbial communities studied with different marker gene assays from the Outokumpu deep biosphere.

Depth m		Dark carbon assimilation	Methanogenesis			Methanotrophy	Nitrate reduction	Sulphate reduction				
		DGGE	cloning	HTP-sequencing		cloning	cloning	DGGE			HTP-sequencing	
Drill hole	Fracture	DNA	DNA	DNA	RNA	DNA	DNA	DNA	RNA	DNA	RNA
	180			Unclassified *mcr*A	n.d						*Desulfatirhabdum*	*Desulfotomaculum*
200		*Parabacteroides*						*Desulfotomaculum*				
		Outoll group						*Pelotomaculum*				
	500			*Methanobrevibacter*	*Methanosarcina*							
								Peptococcaceae	n.d	*Desulfotomaculum*	*Pelotomaculum*	
600		*Parabacteroides*	*Methanosarcina*			*Methylomonas*	*Methylibium*	*Desulfotomaculum*				
								Pelotomaculum				
900		*Parabacteroides*	*Methanosarcina*			*Methylomonas*		*Desulfotomaculum*				
								Pelotomaculum				
	967			n.d	*Methanosarcina*							
								Desulfobulbaceae	*Syntrophobacter*	*Desulfotomaculum*	*Desulfotomaculum*	
1000		*Parabacteroides*					*Pseudomonas*	*Desulfotomaculum*				
								Pelotomaculum				
1100								*Desulfotomaculum*				
1300		*Alkaliphilus*	*Methanolobus*				*Pseudomonas*	*Desulfotomaculum*				
			Methanosarcina				*Methylibium*	*Desulforudis*				
1500		*Alkaliphilus*	*Methanolobus*			*Methylomonas*		*Desulfotomaculum*				
		Outoll group						*Desulforudis*				
1700		*Alkaliphilus*						*Desulfotomaculum*				
		Methanobacterium						*Pelotomaculum*				
	1820			n.d	n.d					*Desulfovibrio*	*Desulfobulbus*	
											Desulfatirhabdum	
1900		*Alkaliphilus*	*Methanobacterium*					*Desulfotomaculum*				
		Methanobacterium						*Pelotomaculum*				
2100		*Alkaliphilus*	*Methanosarcina*					*Desulfotomaculum*				
		Methanobacterium						*Pelotomaculum*				
	2260			*Methanobacterium*	n.d					*Desulfotomaculum*	n.d	
								n.d	n.d	Cand. *Desulforudis*		
	2300	*Alkaliphilus*	*Methanobacterium*	*Methanobacterium*	n.d					n.d	n.d	
		Methanobacterium						*Desulfotomaculum*				
								Pelotomaculum				

n.d = not detected

4.5 Anaerobic respiration in the Outokumpu deep biosphere

The abundance of N_2 gas in the Outokumpu deep subsurface could be a result of microbial denitrification process. Even though the nitrate concentration was below the detection limit in the Outokumpu groundwater, nitrate reducing microbes were present in the drill hole water column. Two types of potential denitrifiers, *Pseudomonas* and *Methylibium* were detected from the depths of 600, 1000 and 1300 m (**Table 9**) (Article II).

Sulfate concentration was typically low, around 1–2 mg l^{-1} in Outokumpu groundwater (Kietäväinen et al. 2013). However, sulfate-reducing microbes were detected both in the fracture zones (Articles I and III) and the drill hole fluids (Article II). The copy number of the marker gene for sulfate reduction, the dissimilatory sulfite reductase *dsrB*, was used as a proxy for the total amount of sulfate reducers in Outokumpu deep bedrock. The *dsrB* copy numbers were in general 300-600 ml^{-1} in the fractures, but in the 500 m fracture the concentration of *dsrB* gene copies was over ten times higher (7.39×10^3 copies ml^{-1}) and in the 967 m fracture over ten times lower (15 copies) compared to the other fracture communities than the one at 500 m depth. Typical members of the sulfate reducing communities in both fracture and drill hole communities were peptococcal phylotypes, such as *Desulfotomaculum* and *Pelotomaculum* (**Table 9**).

4.6 Estimation of the functional potential of predicted archaeal and bacterial metagenomes

The predicted metagenomes of the communities in the different fractures were similar when examined at the top-level functionality. The most abundant genes of the predicted metagenomes were genes involved in metabolism. However, genes involved in amino acid and carbohydrate metabolisms were more abundant in bacteria, while in predicted archaeal metagenomes energy metabolism genes were the most common (Article III) (**Figure 7**). The 180 m fracture zone community differed from the others with lower abundance of genes involved in branched-chain amino acid degradation and fatty acid metabolism. The most abundant energy metabolism genes of the predicted metagenomes of the bacterial communities of the fractures were involved in oxidative phosphorylation. The predicted archaeal metagenomes reflected the pyrosequencing results, as the metagenome of the 967 m fracture differed from the other fractures with higher abundance of genes for amino acid metabolism and low number of genes for energy metabolism. Methane metabolism genes were most abundant in the predicted archaeal metagenomes. In addition, genes needed for biosynthesis of coenzyme M were

present in all predicted archaeal metagenomes. As the methanogenesis pathway from CO_2 and H_2 was present at all depths, methanogenesis from methylamines or methanol was likely operational only in the archaeal communities of the fracture at 180 m (Article III).

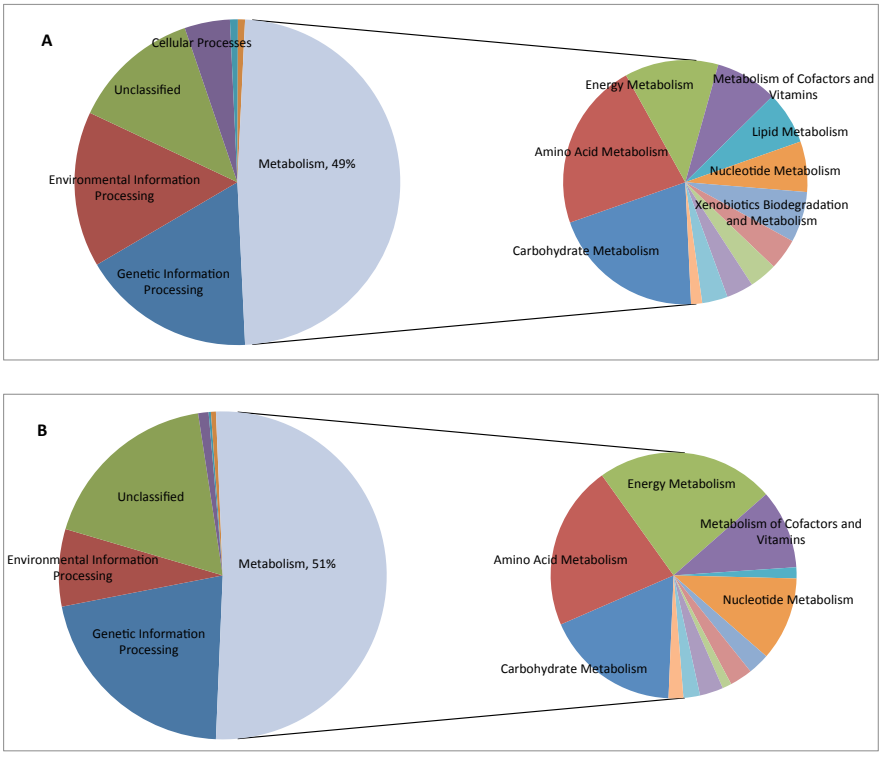

Figure 7. The average abundance of the genes of the predicted a) bacterial and b) archaeal metagenomes.

The nearest sequenced taxon index (NSTI) for predicted bacterial metagenomes ranged from 0.30 to 0.07, meaning that the OTUs of the communities shared on average 70–93% similarity with the reference genomes in the database, respectively. The archaeal NSTIs were lower indicating higher average similarity with reference genomes, except for the total archaeal community at 967 m depth with NSTI 0.29 (**Table 10**).

Table 10. Weighted nearest sequenced taxon indexes for bacterial and archaeal reconstructed metagenomes. The darker the background color of the cell, the higher NSTI value.

Depth	Bacteria		Archaea	
m	DNA	RNA	DNA	RNA
180	0,22	0,10	0,04	0,07
500	0,13	0,07	0,05	0,05
967	0,21	0,10	0,29	0,04
1820	0,07	0,07	n.d	n.d
2260	0,30	0,24	0,04	0,04
2300	0,07	0,12	0,04	n.d

n.d = not detected

4.7 Co-occurrence of OTUs and analysis of the keystone genera of the communities

Approximately 15% of all detected microbial OTUs in Outokumpu deep fracture zones showed significant correlation with each other. The network analysis of the significantly correlating OTUs of the total microbial community divided OTUs in 8 modules with number of nodes ranging from 4 to 41 (**Figure 8**A). The different OTUs in the network were highly connected. According to the high betweenness of centrality value, OTUs affiliating with *Burkholderiales* (*Comamonas*, *Herbaspirillum*, *Pelomonas* and other *Burkholderiaceae*) and with Clostridia (*Desulfitobacter*, other *Clostridiaceae*, *Dethiobacter*) were identified to be the keystone genera of the total microbial community in Outokumpu bedrock. The correlating OTUs of the active microbial community were divided in 8 clusters with number of nodes ranging from 2 to 64 in the network analysis (**Figure 8**B). This network was also highly connected and many of the keystone genera belonged to *Burkholderiales* (*Comamonas, Curvibacter*, other *Oxalobacteraceae* and *Herbaspirillum*).

A

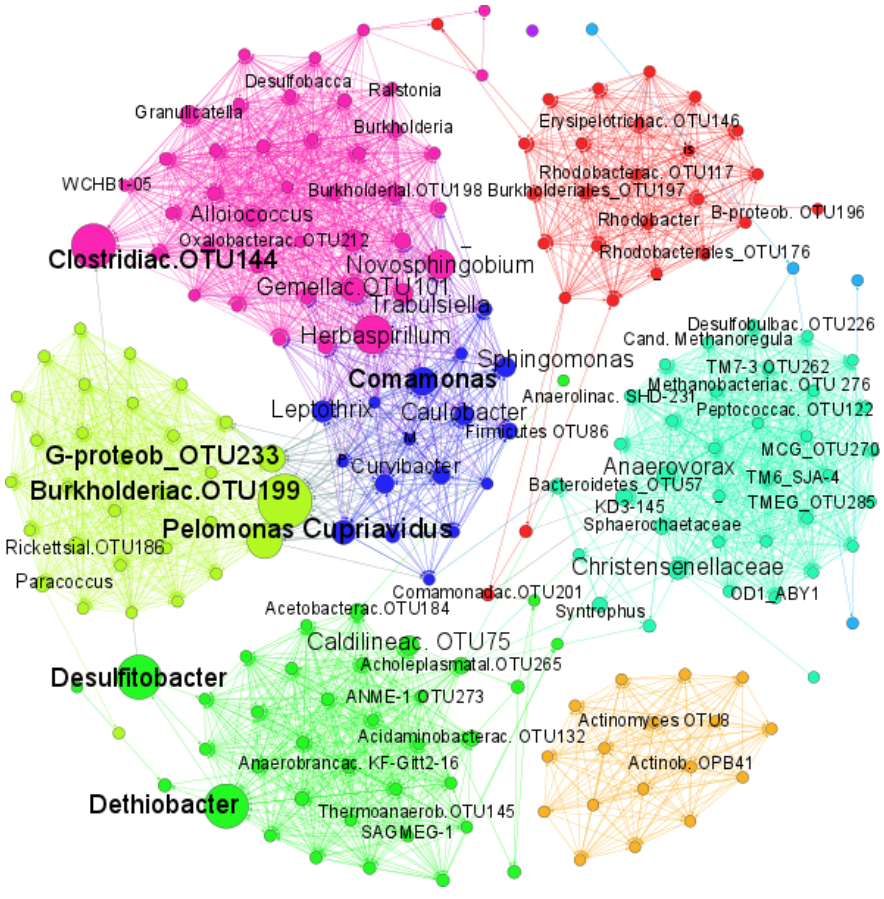

Figure 8. Co-occurrence network of a) the total microbial community and b) the active microbial community in Outokumpu bedrock. Size of the node corresponds to the betweenness of centrality value (Article III).

B

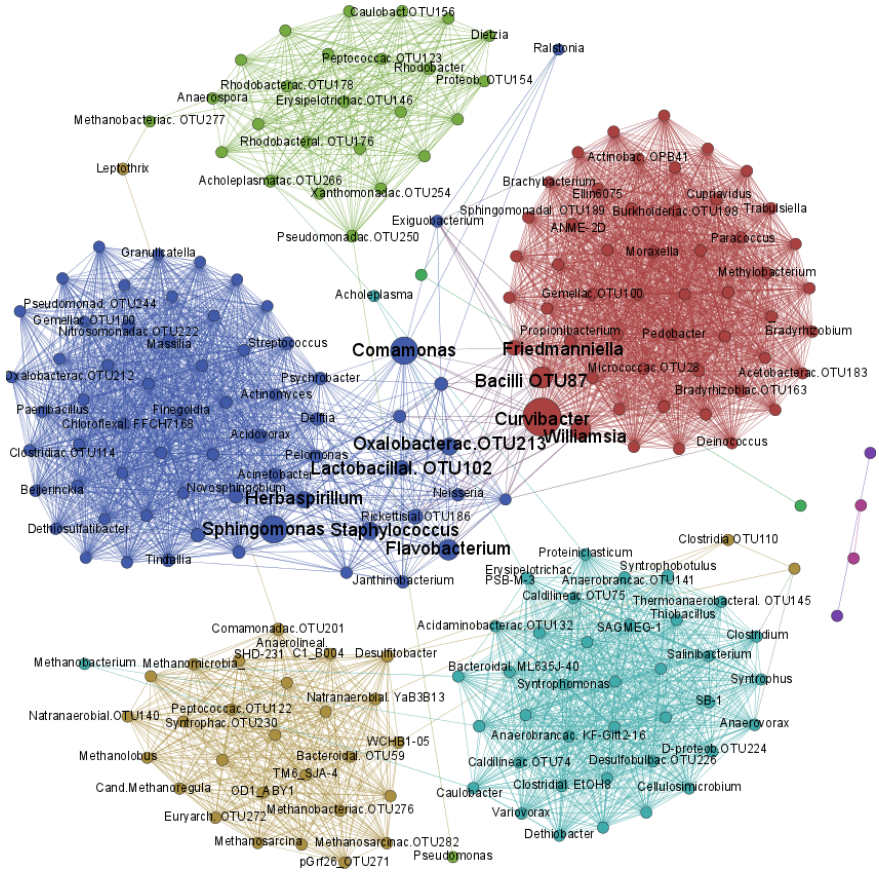

Figure 8. (continued)

5. Discussion

5.1 The microbial communities of the Outokumpu deep biosphere

Cell numbers are low but the diversity is high in Outokumpu deep biosphere. Generally, the most dominant group in the bacterial communities of both the fractures and the drill hole water column affiliate with *Comamonadaceae* (Article III, Itävaara et al. 2011b, Nyyssönen et al. 2014). Other abundant bacterial groups in belong to Clostridia that are commonly detected in deep terrestrial subsurface. Itävaara et al. (2011b) reported over 70% share of Clostridia in the microbial communities in Outokumpu drill hole at 900–1000 and 1400–1500 m. In addition, the highest proportion of clostridial phylotypes throughout the Outokumpu drill hole water column was detected from 1500 m depth (Nyyssönen et al. 2014). Thus, Clostridia are as abundant in fracture zones as they are in drill hole communities between depths of 900 and 1800 m in Outokumpu. Furthermore, the dominant sulfate reducers in Outokumpu fractures and drill hole water column affiliate with clostridial peptococci belonging to *Desulfotomaculum* and *Pelotomaculum* (Article II, III).

Archaea are vertically distributed in Outokumpu fracture zones and drill hole water column. Archaeal communities in the 180 m fracture were the most diverse (Article III). *Methanosarcina* and *Methanolobus* with versatile carbon metabolism in addition to SAGMEG archaea with unknown metabolic properties are more abundant above 1500 m depth in the archaeal communities in Outokumpu. Below this depth, the hydrogenotrophic *Methanobacterium* dominates the archaeal communities in both the fractures and the drill hole water column (Article III, Nyyssönen et al. 2014). Similar distribution is detected with methanogen communities (Article II).

The microbial community structure in the fractures does not reflect the water chemistry at Outokumpu. The bacterial communities in the 180 m and 500 m fractures were highly similar, although the prevalent water type is different. Additionally, the bacterial and archaeal communities in the 500 m fracture are different from the communities in the 967 m fracture albeit the same water type (II). Likewise, the communities in the 1820 m and the 2260 m fractures, both in the vicinity of the water type IV, are dissimilar. The total microbial communities in the 2300 m fracture share features with the microbial communities in the fractures located at shal-

lower depths despite the differences in the water chemistry (Article III, Kietäväinen et al. 2013).

The microbial communities of Outokumpu fractures represent numerous phylotypes with low abundance. These could be regarded as members of the rare biosphere with vast genetic potential to respond to possible environmental changes (Sogin et al. 2006, Article III).

5.2 Comparison of the microbial communities in Outokumpu and other deep terrestrial subsurface environments

The microbial communities in Outokumpu deep crystalline bedrock share common features with other deep ecosystems. The most striking resemblance in bacterial community structure is between Outokumpu fractures and serpentinization-driven environments. Bacteria affiliating with *Comamonadaceae* are dominant in bacterial communities and similar hydrogen-oxidizing clostridial species, such as *Dethiobacter* are detected from Outokumpu fractures, Lost City hydrothermal vents, alkaline spring fluids from Tablelands Ophiolite in USA and from subterrestrial aquifer in Portugal, (Article III, Brazelton et al. 2012, 2013, Tiago and Veríssimo 2013). The co-occurrence analysis revealed that OTUs belonging to *Burkholderiales*, *Clostridiaceae* and *Dethiobacter* were the keystone organisms of the microbial network in deep biosphere of Outokumpu. This further reinforces the importance of the discovery that Outokumpu deep biosphere resembles serpentinization-driven environments and might implicate that carbon and energy substrates formed in serpentinization reactions could sustain the microbial communities in this habitat. The spatial distribution of *Comamonadaceae* and clostridial hydrogenotrophs can be interpreted so that characteristically aerobic *Comamonadaceae* are using hydrogen oxidation in the shallower depths where trace amounts of oxygen can be available, while anaerobic *Dethiobacter* with capacity to reduce sulfur compounds coupled with H_2 oxidation has filled this ecological niche in 967 m fracture. While *Dethiobacter* is unable to reduce sulfate, this fracture also inhabits peptococcal sulfate reducers (*Desulfotomaculum*) in addition to fermenters (such as *Syntrophobotulus* in the active bacterial community).

The core bacterial community in Outokumpu is comprised of few phylotypes, which are also detected in fracture zones at the other Finnish deep bedrock site, Olkiluoto (*Comamonadaceae* and *Pseudomonadaceae*). *Comamonadaceae* are abundant in the bacterial community in the fracture at 600 m depth in Olkiluoto, but otherwise the bacterial communities in Olkiluoto fractures are different from those found from Outokumpu. Likewise, archaeal communities differ significantly in these two Finnish deep biosphere sites. In Outokumpu, archaeal communities are dominated by *Methanobacteriales* but in Olkiluoto, most of the dominant archaea belong to *Thermoplasmatales* and ANME-2D (Bomberg et al. 2014, 2015a,b). *Methanobacteriales* are strictly anaerobic, hydrogenotrophic methanogens, while *Thermoplasmatales* are mainly aerobic heterotrophs, except of the newly described H_2-oxidizing, methanol-reducing methanogens "*Methanoplasma-*

tales" (Paul et al. 2012). Based on the results in Article I and III, the archaeal communities of Outokumpu share features with those in the Witwatersrand deep subsurface (Takai et al. 2001, Moser et al. 2005, Gihring et al. 2006). Hydrogenotrophic CO_2-utilizing methanogens are found from deeper fractures (Takai et al. 2001, Moser et al. 2005, Gihring et al. 2006,) while archaea using more versatile carbon substrates inhabit depths above 1000 m (Lin et al. 2006a, Gihring et al. 2006). In addition, the archaeal community in 967 m fracture in Outokumpu is dominated by SAGMEG archaea, an archaeal candidate division first discovered from and abundant in South African gold mines (Takai et al. 2001). SAGMEG archaea have also been detected from Olkiluoto deep biosphere, from fracture fluids between 400 and 600 m depths (Bomberg et al. 2014, 2015).

Desulfotomaculum are abundant in sulfate reducing communities in Outokumpu and in several South African deep subsurface environments (Baker et al. 2003, Moser et al. 2003, 2005, Trimarco et al. 2006, Silver et al. 2010). This implies that the similar environmental conditions in these isolated deep subsurface environments favor especially sulfate reducers that are *Desulfotomaculum* -type of nonacetate oxidizers.

5.3 Inorganic energy substrates

In Outokumpu anaerobic deep subsurface, the possible energy conservation processes include nitrate, iron, manganese and sulfate reduction.

Potential for nitrate reduction was detected from drill hole water column from 1300 m and above (Article II). The *narG*-marker gene clones affiliated with *Pseudomonas*, which was one of the keystone genera in Outokumpu. *Pseudomonas* represents a typical denitrifier coupling organic carbon oxidation to nitrate reduction. In terrestrial deep bedrock biosphere of Äspö and other Swedish sites, nitrate reducers are the dominating physiological groups in addition to acetogens and sulfate reducers (Hallbeck and Pedersen 2008a, 2012). While some iron and manganese reducers have been detected in Äspö, very few or none of these taxa were detected in the community analyses in Outokumpu.

The traditional sulfate-reducing bacteria represent only a fraction of the total bacterial community. On the other hand, the total bacterial community structure suggests that thiosulfate reduction is a major metabolic trait in the fracture communities at 967 m and 1820 m depth with *Dethiobacter* and *Dethiosulfatibacter* as the dominating taxa. Neither of these thiosulfate-reducing bacteria can use sulfate as an electron acceptor (Sorokin et al. 2008, Takii et al. 2007). QPCR results confirm that in the 967 m fracture where *Dethiobacter* that does not possess *dsr*AB gene (Sorokin et al. 2008), was particularly abundant, the copy numbers of the *dsrB* gene were extremely low. Interestingly, *Syntrophobotulus* that was dominating the active bacterial community in the 967 m fracture is described to be a chemotrophic fermenter of glyoxylate but may also be the fermenting partner in a syntrophic relationship with methanogens or acetogens producing hydrogen and carbon dioxide for these microbial groups. The active archaeal and methanogen

communities in this fracture comprised of *Methanobacterium* and *Methanosarcina*, both of these can use hydrogen and carbon dioxide produced by *Syntrophobotulus*. The metagenomic data suggested that genes involved in acetyl-CoA fermentation and synthesis of acetone-ethanol-butanol at 1500 m depth in the drill hole (Nyyssönen et al. 2014). This depth also represented the highest proportion of clostridial phylotypes in the drill hole. The 1820 m fracture hosts the richest active sulfate reducing community and the copy numbers of *dsrB* gene transcripts were among the highest detected throughout the Outokumpu deep subsurface. Evidence for sulfate reduction can be sensed from the fluids especially at 1820 m depth, where the water has the distinctive odor of hydrogen sulphide.

5.4 Autotrophic potential vs. heterotrophy

The model for primary production in deep terrestrial biosphere is based on hydrogen and CO_2. Autotrophic organisms, such as acetogens or methanogens use the reducing power of hydrogen to produce acetate or methane from carbon dioxide (Pedersen 1997, 2000). However, organic compounds in deep subsurface can provide both carbon and energy source for heterotrophic microorganisms (Amend and Teske 2005, Colwell and D'Hondt 2013, Schrenk et al. 2013).

Many microbial groups abundant in Outokumpu fractures are able to use CO_2 as a carbon source (e.g. *Comamonadaceae*, *Dethiobacter*, *Desulfotomaculum*, *Methanobacterium*) (Article I, II, III). *Comamonadaceae* dominating the bacterial communities especially in the fracture zones at 180 and 500 m is a metabolically diverse group of organisms. *Comamonadaceae* are either chemoorganotrophs with a capacity to use a range of organic acid substrates or H_2-oxidizing aerobic chemolithotrophs (Willems et al. 1991). Clostridia were also frequently detected in microbial communities in Outokumpu (Articles I, II, III, Itävaara et al. 2011a,b, Nyyssönen et al. 2014). Many acetogens, autotrophic microbes producing acetate from CO_2 via autotrophic Wood-Ljungdahl pathway, belong to Clostridia. Acetogenesis has been demonstrated to have a significant role in the deep biosphere in Forsmark and Laxemar, Sweden, where acetogens were dominating the microbial communities (Hallbeck and Pedersen 2012). Bomberg et al. (2015b) detected genes coding the enzymes of Wood-Ljungdahl pathway in predicted bacterial metagenomes in Olkiluoto fractures in Finland, among other major autotrophic CO_2 fixation pathways. However, the specific marker gene for Wood-Ljungdahl pathway could not be detected with PCR from the Outokumpu drill hole (Article II) and the relative abundance of autotrophic CO_2 fixation mechanisms including the Wood-Ljungdahl pathway in the predicted bacterial metagenomes of the Outokumpu fractures was low (Article III). Conversely, in a metagenomic study of Outokumpu drill hole fluids, autotrophic reduction of CO_2 to acetate was evident (Nyyssönen et al. 2014). Nevertheless, as another autotrophic pathway, Calvin-Bassham-Benson cycle could not be verified either with the marker gene approach and *acc*C genes affiliating mainly with heterotrophic microbes were detected from all studied drill hole depths (Article II), it can be concluded that chemoor-

ganotrophs are major players in CO_2 assimilation in deep biosphere of Outokumpu (Article II). Incorporation of CO_2 via carboxylation reactions is important to heterotrophs for compensation of the metabolic imbalance created with utilization of carbon in biosynthesis and anaplerotic reactions. CO_2 incorporation by heterotrophs may be an important survival mechanism in oligotrophic, nutrient-depleted conditions (Alonso-Sáez et al. 2010) that also prevail in Outokumpu deep subsurface.

Some of the sulfate reducers detected in Outokumpu are facultative chemolithoautotrophs using Wood-Ljungdahl pathway for biosynthesis of organic compounds from CO_2, but many of these organisms can also use fermentation of organic compounds such as lactate, pyruvate and ethanol for energy and carbon source. *Desulfotomaculum*, *Desulfovibrio* and *Desulfobulbus* that were members of the sulfate reducing communities in Outokumpu fractures are characterized as nonacetate oxidizers, i.e. they excrete acetate as an end product (Madigan et al. 2008). Thus, these can provide a suitable carbon source for many heterotrophic microorganisms in Outokumpu deep biosphere.

Hydrogenotrophic methanogens are abundant members of the archaeal communities in Outokumpu, but as the number of archaeal cell number is only a fraction of bacteria (0.5% on average) based on 16S rRNA gene quantification (Article III), methanogens may not produce enough biomass to feed the heterotrophic bacterial communities. In addition, although marker genes of methane oxidation were detected in Outokumpu (Article II), known methane oxidizers were not among the most abundant taxa in the microbial communities.

Autotrophs are dependent on CO_2. However, the CO_2 concentration in Outokumpu deep bedrock is very low (Kietäväinen et al. 2013). This indicates that the CO_2 flux from other parts of the bedrock is either not very rapid and/or only a fraction of it enters the studied fractures while the gross of it has been consumed elsewhere. Since CO_2 is not abundantly available and autotrophic CO_2 fixation pathways could not be verified, the microbial communities in Outokumpu were presumed to use mainly organic carbon compounds. The classification of the bacterial community data on family level and determining the predominant metabolism of each family according to The Prokaryotes (Rosenberg et al. 2013) showed that organotrophy is the most common resolved metabolic trait in Outokumpu fracture zone communities (fracture zone communities (**Figure 9**) (Article III). However, in deeper fractures and especially in the active communities, several bacterial OTUs could not be classified to family level and thus their metabolic properties remain unclear.

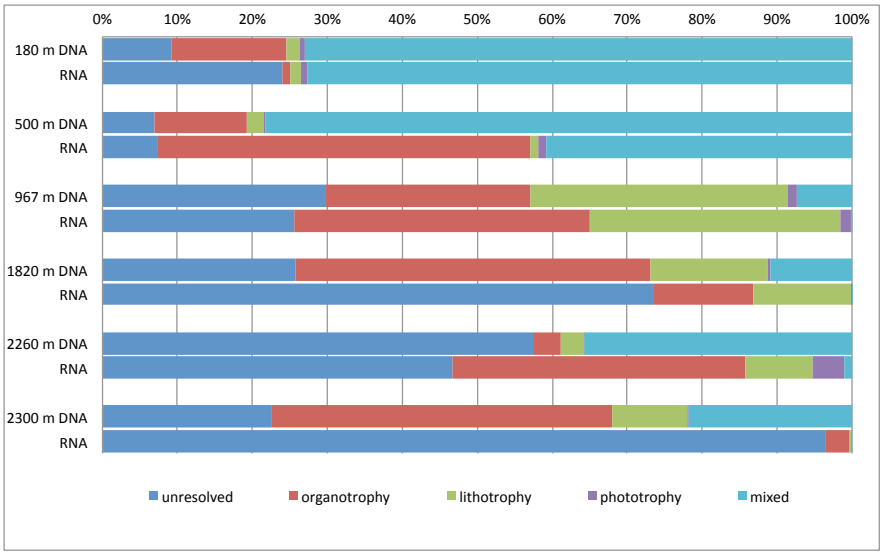

Figure 9. Bacterial physiotypes of OTUs in different fracture zone communities classified by their apparent metabolism (according to The Prokaryotes).

These results suggest that autotrophy may not be as common in Outokumpu as it is for example in Fennoscandian deep subsurface sites in Sweden (Pedersen 2000, Hallbeck and Pedersen 2012) and in Olkiluoto, Finland (Bomberg et al. 2015b).

5.5 Plausible geochemical organic carbon sources in Outokumpu

The drill hole pierces through the Outokumpu assemblage at the depth of 1300-1500 m (**Figure 2**). This assemblage contains ophiolitic rocks such as serpentinite, diopside and tremolite skarn (Västi 2011). This rock sequence can be the source of small organic carbon compounds that are known to be present in serpentinizing environments (McCollom and Seewald 2001, Lang et al. 2010, Lang et al. 2012). The seismic reflectors of the area indicate that this is not the only depth where ophiolite-derived rocks are located in Outokumpu area (Heinonen et al. 2011). For example, in the vicinity of the drill hole near 1000 m depth, a thin lens of ophiolitic rocks can be located in addition to the expansion of the main ore belt of the Outokumpu formation at depths above 200 m. Furthermore, size, volume and direction of the fracture zones are not known, thus some of these might reach these ophiolitic rock sequences. High microbial diversity in addition to high proportion of chemoorganotrophic clostridia at 1500 m depth in the drill hole (Nyyssönen et al. 2014) suggests that especially the small organic compounds formed in reducing conditions triggered by the serpentinization of ophiolitic rocks may provide

a prominent carbon source for the heterotrophic microbial communities. This might also be true for the microbial communities at 967 m and 1820 m fracture zones.

The lithology of Outokumpu Deep Drill Hole comprises of several black schist layers ranging from 0.1 to 13 m in thickness (Västi 2011). These were formed from organic matter deposited in anoxic seawater basins c. 1.9–2.0 Ga ago (Loukola-Ruskeeniemi 2011). Graphite in abundant black schist layers of mica gneiss in Outokumpu contains trace quantities of hydrocarbons (Taran et al. 2011). There are several reports on microbes using kerogen-containing black shale as sole carbon source, and these affiliate to *Clostridia* and *Pseudomonadales* (Petsch et al. 2001, 2005, Rosewarne et al. 2013). Both of these represent components of the Outokumpu core microbial community, although the abundance of these groups varies in the communities. In addition, clostridial phylotypes represent keystone species in Outokumpu deep biosphere. Fermenting clostridia could use the organic carbon derived from black schist in Outokumpu. In addition, some *Pseudomonas* species have the ability to degrade hydrocarbons in anaerobic conditions (Chayabutra and Ju 2000, Lalucat et al. 2006). By degrading complex organic materials, *Pseudomonas* in Outokumpu may provide more utilizable substrates for the rest of the microbial community. However, further proof is needed to demonstrate whether the microbial utilization of the recalcitrant, ancient carbon source of black schist graphite is possible in Outokumpu.

5.6 Evaluation of the used microbial community characterization methods

Microbial community changes can be tracked with fingerprinting methods such as DGGE. Thus, this method was chosen in Article I to evaluate the effect of the pumping period to the microbial community and also to get a first impression on the microbial community composition in the intrinsic fracture fluids in Outokumpu. This study demonstrated that an extensive pumping period was needed for attaining the intrinsic fracture fluid for microbial community analyses. DGGE revealed several bacterial, archaeal and sulfate reducer phylotypes. However, DGGE is limited in detecting only the dominant species of the microbial community, also in deep terrestrial biosphere (Muyzer et al. 1993, Kirk et al. 2004, Bomberg et al. 2014). The more detailed characterization of the microbial communities of the fractures was made with high-throughput sequencing (Article III). With this method, the rare biosphere and the co-occurrence network patterns of the microbial communities in deep terrestrial bedrock could be revealed. The number of bacterial phylotypes detected with DGGE in the fracture communities was almost a hundred times lower in most cases compared to the number of observed species from HTP data (**Table 11**). DGGE phylotypes of archaeal and sulfate reducing communities on the other hand covered more of the diversity. In the 500 m fracture, a double amount of archaeal phylotypes was detected with DGGE in comparison to HTP sequencing, probably because of the low amount of archaea (< 100 16S

rRNA gene copies ml^{-1}) in the sample affected to the amplification efficiency (Article III).

Table 11. Number of the detected phylotypes with DGGE and HTP sequencing in three fractures.

Fracture depth m		Characterization method			
		DGGE fingerprinting		HTP sequencing	
		DNA	RNA	DNA	RNA
500	bacteria	11	13	329	396
	archaea	7	8	3	3
	SRB	4	n.a	51	7
967	bacteria	10	15	274	318
	archaea	20	8	58	30
	SRB	7	5	25	12
2260	bacteria	6	4	286	446
	archaea	9	9	7	9
	SRB	n.a	n.a	37	n.a

n.a = not available

The DGGE analysis did perform well for rough detection of the dominating microbial groups, such as β-proteobacteria in 500 m fracture zone and *Clostridia* in deeper zones along with SAGMEG in 967 m fracture and *Methanobacteriales* dominating in other archaeal communities. Nevertheless, with over a hundred times more bacterial phylotypes detected by HTP sequencing in this study, the HTP sequencing method revealed the high diversity of the community more efficiently.

Functional communities were characterized with DGGE or clone libraries. Clone libraries based on nitrate reduction and methane oxidation marker genes revealed only few different phylotypes, while the *mcr*A clone library reflected fairly well the archaeal HTP sequencing results of the drill hole communities (Nyyssönen et al. 2014). Phylotypes affiliating with *Methanobacterium* were dominant in both clone libraries and pyrosequences at 1900 and 2300 m depth, while *Methanolobus*-, *Methanobacterium*- and *Methanosarcina* -affiliating clones were found at 1300 m depth in both studies. However, methanosarcinal phylotypes dominated the methanogen clone libraries at 900 m and above in the drill hole, but in the fracture zone communities, this group was represented a low relative abundance and was present only in the 180 m fracture. Hence, specific functional gene clone libraries may be sensitive enough to detect small but significant functional microbial groups.

6. Conclusions

Outokumpu fracture zones host diverse microbial communities depending mainly on chemoorganotrophic metabolism. These communities are intrinsic, i.e. they differ in structure from both the drill hole microbial communities and from fracture to fracture. Bacteria form the majority of these communities, but several archaeal taxa and different subcommunities based on functionality were detected. Most dominant phylotypes in the microbial communities belong to β-proteobacteria and *Clostridia*, particularly to *Comamonadaceae*, *Peptococcaceae* and *Anaerobrancaceae*. Representatives of these are the keystone genera of the Outokumpu fracture ecosystem. Many OTUs detected in this study were affiliated with phylotypes or species detected from serpentinization-driven ecosystems and other deep subsurface environments.

Hydrogenotrophic metabolism is characteristic to the most dominant microbial groups, but the organic carbon compounds usage infers that heterotrophy is important carbon assimilation strategy in deep subsurface on Outokumpu. In addition, autotrophy might play only a minor role in carbon fixation in deep subsurface of Outokumpu, as marker genes for common autotrophic carbon fixation pathways were not detected.

In summary, this thesis contributes to the biogeographic trend that different deep subsurface sites host microbial communities with structural similarities often related with depth. The geochemical properties of the deep subsurface appear to determine the microbial community structure to a certain level. The carbon and energy sources are likely the most important factors determining the community structure in Outokumpu.

7. Future outlook

Microbial communities have been characterized successfully from several terrestrial deep subsurface sites. With the aid of HTP sequencing methods and subsequent computational bioinformatics analyses and data mining methods, in addition to traditional enrichment or cultivation-based approaches, the functionality of the microbial communities is unveiling. However, there are several questions remaining that need to be addressed in future research of deep crystalline bedrock biosphere. For example, how deep can we still find life in the Earth's crust? How have microbes adapted to the long-term isolation and the environmental stress? How long have microbes existed in the depths? Distinguishing the environmental factors that hinder the existence of microbial life in depths of bedrock would allow us to determine the ultimate limits of life. In addition, answering these questions might bring us closer to understanding the origin of life on Earth and possibilities of life on other celestial bodies such as Mars.

Another question concerns the possible sources of carbon and other chemical elements imperative to life in deep bedrock biosphere. Geochemical processes yielding both soluble organic and inorganic carbon are relatively well known, but microbial contribution to these processes is not well understood in deep environments. In addition, microbial utilization of solid materials, such as rocks containing recalcitrant carbon compounds would need more research. Moreover, the occurrence and influence of serpentinization in Outokumpu remains unclear. On-going serpentinization is not reported in Outokumpu, even though ophiolitic rocks are abundant, so the hypothesis of serpentinization-driven microbial communities remain unsupported until the residence times of the products, the movements in the bedrock and flux from the origin of serpentinization are resolved.

Lastly, there are recent studies on eukaryotes inhabiting marine and terrestrial deep biosphere (Orsi et al. 2013, Sohlberg et al. 2015). These have not been characterized from Outokumpu, even though for example some fungi are known for their ability to degrade complex carbon compounds found in rocks. This capacity might be a key process for releasing the carbon from complex, recalcitrant material of black schist for the use of the rest of the microbial community.

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Title	**Microbial ecology and functionality in deep Fennoscandian crystalline bedrock biosphere**
Author(s)	Lotta Purkamo
Abstract	Microbial life in the deep subsurface contributes significantly to overall biomass on Earth. Although the microbial communities inhabiting the deep subsurface are abundant, little is known about their diversity, activity, interactions and role in global biogeochemical cycles. The diversity of microbial life in the deep terrestrial subsurface of the Fennoscandian shield was studied with molecular biological methods. The Outokumpu Deep Drill Hole provides access to crystalline bedrock fluids that are estimated to be tens of millions of years old. Characterization of the indigenous bacterial and archaeal communities in addition to microbial communities with important functional properties in bedrock fluids was done from a depth range of 180 m to 2300 m. Microbial community profiling and assessment of possible functional processes was done with molecular fingerprinting, cloning and sequencing methods combined with suitable statistical and bioinformatics analyses. Low cell numbers but high diversity was characteristic to the microbial communities of the Outokumpu deep subsurface. The microbial communities in the fracture zones had in general fewer cells than those in the mixed fluids of the drill hole. *Comamonadaceae*, *Peptococcaceae* and *Anaerobrancaceae* were prevalent bacterial members of the microbial communities in the fracture fluids. Archaea were a minority in microbial communities. Sulfate-reducing bacteria and methanogens were detected at several depths. Microbial communities resembled those detected from other deep Fennoscandian Shield subsurface sites. Furthermore, sulfate reducing communities and archaeal communities resembled those found from the deep subsurface of South Africa. Investigation on carbon assimilation strategies of the microbial communities revealed that mainly heterotrophic *Clostridia* were responsible for CO_2 fixation in this habitat. Representatives of *Burkholderiales* and *Clostridia* formed the core microbial community and these were also identified to be the keystone genera. The microbial communities of Outokumpu fractures share similarity with those of serpentinization-driven ecosystems. Energy and carbon substrates formed in serpentinization reactions of ophiolitic rocks in Outokumpu may sustain the microbial communities in this deep subsurface environment.
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Nimeke	**Mikrobien ekologia ja toiminnallisuus syvällä Fennoskandian kiteisessä kallioperässä**
Tekijä(t)	Lotta Purkamo
Tiivistelmä	Syvällä maanpinnan alla elävät mikrobit muodostavat merkittävän osan maapallon kokonaisbiomassasta. Vaikka syvällä elää runsaasti mikrobeja, niiden monimuotoisuudesta, aktiivisuudesta, vuorovaikutuksista ja roolista maailmanlaajuisissa biogeokemiallisissa kierroissa tiedetään vielä vähän. Mikrobielämän monimuotoisuutta Fennoskandian kilven kallioperässä tutkittiin käyttäen molekyylibiologisia menetelmiä. Outokummun syvä kairareikä tarjoaa mahdollisuuden tutkia kiteisen kallioperän vesiä, joiden on arvioitu olevan kymmeniä miljoonia vuosia vanhoja. Kallioperän endeemisten bakteeri- ja arkeoniyhteisöjen koostumusta ja merkittäviä toiminnallisia ominaisuuksia luonnehdittiin syvyysvälillä 180–2300 m. Mikrobiyhteisöjen toiminnallisten ominaisuuksien arviointi toteutettiin molekyylibiologisin menetelmin käyttäen sormenjälki-, kloonaus- ja sekvensointimenetelmiä sekä sopivia tilastollisia ja bioinformatiikan analyysejä. Matala solumäärä mutta korkea monimuotoisuusaste leimasivat mikrobiyhteisöjä Outokummun syvässä kallioperässä. Rakovyöhykkeissä oli yleisesti vähemmän soluja kuin sekoittuneessa kairareiän vesipatsaassa. *Comamonadaceae*-, *Peptococcaceae*- ja *Anaerobrancaceae*-sukujen bakteerit olivat vallitsevia rakovyöhykkeiden mikrobiyhteisöissä. Arkeonit olivat vähemmistönä yhteisöissä. Sulfaattia pelkistäviä bakteereita ja metaanintuottajia havaittiin useissa eri syvyyksissä. Mikrobiyhteisöt muistuttivat muista Fennoskandian syvistä ympäristöistä havaittuja mikrobiyhteisöjä. Lisäksi sulfaatinpelkistäjät sekä arkeoniyhteisöt olivat samankaltaisia Etelä-Afrikan syvän kallioperän mikrobiyhteisöjen kanssa. Mikrobien hiiliaineenvaihdunnan tutkimus paljasti heterotrofisten klostridien toimivan pääasiallisina hiilidioksidin sitojina näissä elinympäristöissä. *Burkholderiales*- ja *Clostridia*-bakteeriryhmien edustajat muodostivat nk. Outokummun kallioperän ydinmikrobiston, eli näiden ryhmien jäseniä löytyi kaikista syvyyksistä. Lisäksi näiden ryhmien edustajien todettiin olevan yhteisöjen avainlajeja. Outokummun kallioperän rakovyöhykkeiden vesissä elävät mikrobiyhteisöt ovat samankaltaisia kuin ne yhteisöt, joita löytyy ekosysteemeistä joiden energia ja hiili ovat lähtöisin ofioliittisten kivien serpentinisaatiosta. Serpentinisaatioreaktioissa muodostuvat energian- ja hiilenlähteet voivat ylläpitää mikrobiyhteisöjä myös Outokummun syväbiosfäärissä.
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Microbial ecology and functionality in deep Fennoscandian crystalline bedrock biosphere

The need for a comprehensive understanding of the microbial processes in deep subsurface and the environmental factors regulating these activities has recently been growing simultaneously with the industrial interest of utilization of deep bedrock for material or energy source or storage space. In addition to the assessment of the microbial risks of these operations, microbial ecological research of the deep subsurface may provide innovations for different types of industries in form of novel microbes, enzymes or bioactive compounds. Furthermore, research on microbial ecology of the deep subsurface will improve our ability to understand the origin of life on Earth and possibility of extraterrestrial life on other planetary bodies.

The microbial communities in Outokumpu deep crystalline bedrock fluids share common features with other deep ecosystems.Thus, this thesis contributes to the biogeographic trend that different deep subsurface sites host microbial communities with structural similarities often related with depth. The geochemical composition and the sources of carbon and energy in the deep, ancient fluids in bedrock are likely the most important factors determining the community structure in Outokumpu subsurface.

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